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STUDIES ON NEUROTROPIC RIFT VALLEY FEVER VIRUS: THE SUSCEPTIBILITY OF SHEEP AND MONKEYS.

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IN previous communications (Mackenzie and Findlay, 1936; Mackenzie, Findlay and Stern, 1936) an account was given of the production in mice of a neurotropic strain of the virus of Rift Valley fever and of its behaviour in rodents. In the present paper the pathogenic action of neurotropic Rift Valley fever virus in sheep, lambs and monkeys is described.

THE SUSCEPTIBILITY OF SHEEP AND LAMBS.

The original discovery of Rift Valley fever virus by Daubney, Hudson and Garnham (1931) was due to the occurrence of a severe epizootic that occurred in 1930 in the flocks of sheep and lambs pastured on the shores of Lake Naivasha in Kenya, British East Africa. Both in the field and in the laboratory the virus isolated was shown to be highly pathogenic for lambs, causing death in from 36 to 48 hours after inoculation, with widespread necrosis of the liver. In adult sheep the action of the virus was somewhat less virulent, but those still pregnant almost invariably aborted and the mortality was about 20 p.c. The liver necrosis, instead of being widespread, was focal in character. Other lesions found in sheep and lambs were swelling of the mesenteric lymph-nodes, congestion and sometimes hæmorrhage in the gastric and intestinal mucosa, subendocardial and subepicardial ecchymoses, and degenerative changes in the tubular cells of the kidney. These results were confirmed by Findlay (1931–32), who found that the pathogenicity for sheep and lambs was preserved after passage through mice.

The behaviour of the neurotropic variant was studied in four lambs from 3 to 4 weeks old, and in two adult ewes of the Southdown breed.

Two lambs, 1 and 2, were each inoculated intraperitoneally with 5 c.c. of a 20 p.c. suspension of mouse brain infected with neurotropic Rift Valley virus after 25th and 28th passages in mouse brain. The temperature rose within 24 hours of inoculation from 102·8° to 105·4° F. in lamb 1

and from 103·4° to 105·2° F. in lamb 2. In both animals the temperature had returned to normal within 96 hours of inoculation. During this period virus could still be obtained from the circulating blood, as shown by intracerebral inoculation of mice, but the lambs showed no sign of illness, and continued to feed and move about actively. Blood was removed from both lambs 10 days after inoculation, and tested for immunity by injecting mixtures of undiluted serum and falling dilutions of pantropic virus intraperitoneally

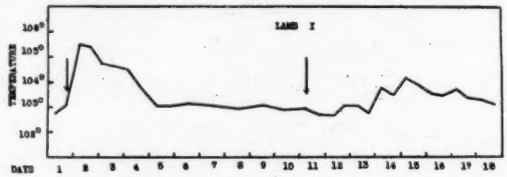

Chart 1.—Temperature reaction of lamb 4 following intracerebral injection of neurotropic Rift Valley fever virus.

into mice. The sera of both lambs were found to protect mice against 1000 M.L.D. of pantropic Rift Valley fever virus. In the case of lamb 1, as shown in Chart 1, pantropic virus in the shape of 10 c.c. of a 1 in 10 dilution in saline of infected mouse blood was inoculated intraperitoneally 11 days after injection of the neurotropic virus; a slight but hardly significant rise of temperature occurred 3 and 4 days later, but no free virus was obtainable from the blood during this febrile period. Twenty-one days after inoculation the immunity

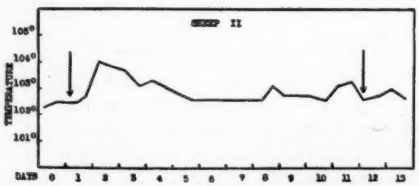

Chart 2.—Temperature reaction of sheep 2 following intraperitoneal injection of neurotropic Rift Valley fever virus (1st arrow) and pantropic Rift Valley fever virus (2nd arrow).

was again tested; the blood-serum now protected against 1,000,000 M.L.D. of pantropic Rift Valley fever virus. Similar results were obtained with lamb 2, though in this case the injection of pantropic virus was not made till 14 days after the injection of neurotropic virus, and the serum was tested for immunity 3 weeks after the original injection and found to neutralize 10,000,000 M.L.D.

Two adult sheep, each inoculated intraperitoneally with 5 c.c. of a 20 p.c. suspension of mouse brain infected with the neurotropic strain of Rift Valley fever virus (28th and 32nd passages in mouse brains) showed a slight and temporary rise of temperature 24 hours after inoculation (Chart 2). Virus was present at this period in the peripheral blood-stream, but had disappeared by the 5th day. The sheep showed no signs of ill-health during this period, and their appetites were unimpaired. Fourteen and 12 days after inoculation the

Chart 1.—Temperature reaction of lamb 1 inoculated as for sheep 2.

sheep were injected intraperitoneally with 10 c.c. of mouse blood infected with pantropic Rift Valley fever virus. No febrile reaction followed this injection, there was no evidence of ill-health, and free virus was not obtained from the peripheral blood-stream. The sera of the two sheep, tested in mice as before for immunizing power 21 and 26 days after injection, were found capable of neutralizing 1,000,000 M.L.D. of pantropic virus. There is thus evidence that a high degree of immunity against Rift Valley fever may be obtained both in sheep and lambs by a preliminary injection of the modified neurotropic strain of the virus, followed later by inoculation of the pantropic virus.

Before this method of immunization can be undertaken in the field it must obviously be tested on a more extensive scale. Mouse brains infected with neurotropic Rift Valley fever virus have, therefore, after freezing and drying, been despatched to Kenya, where they are being tested more extensively in sheep and lambs by Mr. R. Daubney, Chief Veterinary Research Officer. Judging from results obtained in mice one of the possible dangers in the intra-peritoneal or subcutaneous inoculation of neurotropic Rift Valley fever virus, at any rate into young lambs, lies in the possibility that the virus may reach the central nervous system, where, as the following experiments show, it may cause a rapidly fatal encephalitis.

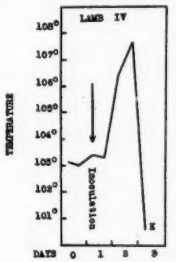

Chart 3.—Temperature reaction oflamb 2 inoculated as for sheep 2.

Lamb 3 was inoculated intracerebrally with 1 c.c. of a 20 p.c. suspension of mouse brain infected with neurotropic Rift Valley fever virus that had undergone 32 passages in mouse brains. The temperature at inoculation was 102·8° F. Twenty-four hours later the temperature had risen to 105·0° F., but the lamb showed no abnormal symptoms ; next morning it was found dead. At the post-mortem there were no naked-eye lesions of the liver, mesenteric lymph-nodes, heart or abomasum. The distribution of virus is shown in Table I.

Lamb 4 was also inoculated intracerebrally with 0·75 c.c. of a 20 p.c. suspension of mouse brain infected with neurotropic virus that had undergone 34 passages in mouse brains. The temperature changes are seen in Chart 3. Forty-eight hours after inoculation the lamb was unable to rise from the ground, the head was retracted, and there were convulsive twitchings of the limbs. The animal was killed ; no abnormal lesions were found *post mortem*. The distribution of virus in the tissues was tested by the intracerebral and intraperitoneal injection of batches of 6 mice.

TABLE I.—*Distribution of Virus in the Tissues of Lambs Dying of Neurotropic Rift Valley Fever.*

	Blood.		Liver.		Spleen.		Brain.	
	intra-cerebral.	intra-peritoneal.	intra-cerebral.	intra-peritoneal.	intra-cerebral.	intra-peritoneal.	intra-cerebral.	intra-peritoneal.
Lamb 3	0/6	0/6	0/6	0/6	0/6	0/6	6/6	1/6
Lamb 4	0/6	0/6	0/6	0/6	0/6	0/6	6/6	2/6

Chart 3.—Temperature reaction of lamb 4 following intracerebral injection of neurotropic Rift Valley fever virus.

Of the mice dying after injection of brain tissue only an occasional one showed slight signs of liver necrosis, while all developed symptoms of cerebral involvement with encephalitic lesions in the brain.

The distribution of virus in these lambs is thus very different from that found after injection with viscerotropic virus, nor is there any evidence that as a result of passage through the lamb the neurotropism for the mouse had been decreased.

THE SUSCEPTIBILITY OF MONKEYS.

The susceptibility to ordinary Rift Valley fever of a number of different species of monkey was investigated by Findlay and Daubney (1931) and Findlay (1931–32 and 1932–33). It was found that the injection, either subcutaneously,

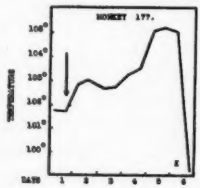

FIG. 4.

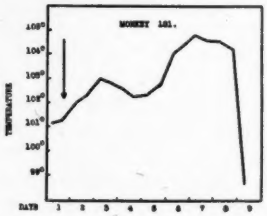

FIG. 5.

Chart 4.—Temperature reaction of monkey 177 following intracerebral inoculation of neurotropic Rift Valley fever virus.

Chart 5.—Temperature reaction of monkey 181 following intranasal instillation of neurotropic Rift Valley fever virus.

intraperitoneally, intracerebrally or by nasal instillation, gave rise after a short incubation period to a brief febrile reaction, from which the monkeys invariably recovered. Virus was present, however, for some days in the peripheral blood-stream before the development of immune bodies, while in the liver there were small scattered areas of focal necrosis. The neurotropic strain of Rift Valley fever, however, was invariably fatal when introduced intracerebrally or by intranasal instillation. Intraperitoneal inoculation alone was followed by no definite febrile reaction, but if combined with the intracerebral inoculation of starch a fatal encephalitis invariably developed. Two species of monkey were found to be highly susceptible to neurotropic Rift Valley fever, for though the rhesus monkey *Macaca mulatta* was chiefly employed, the crab-eating macacque, *M. irus*, was also found to succumb to encephalitis. Details of the monkeys inoculated are recorded in Table II.

Typical protocols are as follows :

Rhesus monkey 177, inoculated intracerebrally November 4th, 1935, with 1 c.c. of a 20 p.c. suspension in saline of mouse brain infected with neutropic Rift Valley fever virus, after 12 passages in mouse brain. Four days after inoculation the temperature rose to 105° F. On the following day the temperature varied from 105° to 105·2° F.; weakness began to develop in the hind legs and quickly became more pronounced. On the sixth day after inoculation the temperature had fallen to 99·2° F., and the monkey, which was so weak that it could not rise from the floor

of its cage, was killed (Chart 4). Seventy-two hours after inoculation blood was removed from the heart and inoculated intracerebrally into a batch of 6 mice; at death tests were again made for the presence of virus in the heart blood and in the liver, spleen, adrenal and brain by intracerebral inoculation of mice; the results are shown in Table II.

TABLE II.—*Monkeys Inoculated with the Virus of Neurotropic Rift Valley Fever.*

No.	Species.	Inoculum and route of inoculation.	Number of days from inoculation to death.	Remarks.
176	*M. mulatta*	0·75 c.c. Berkefeld V filtrate, mouse brain, 10th passage, i.c.	5	Encephalitis.
177	„	1 c.c. 20 p.c. suspension, mouse brain, 12th passage, i.c.	6	„
179	„	0·75 c.c. 20 p.c. suspension, monkey 176, i.c.	6	„
180	„	5 c.c. 20 p.c. suspension, mouse brain, 20th passage, i.p.	Survived	No febrile reaction.
181	„	1 c.c. 20 p.c. suspension, mouse brain, 15th passage, intranasal instillation	8	Encephalitis.
182	„	Mouse brain, 25th passage, intranasal instillation	9	„
187	„	5 c.c. 20 p.c. suspension, brain of monkey 181, i.p.; 1 c.c. 2 p.c. starch i.c.	21	„
189	„	5 c.c. 20 p.c. suspension, brain of Monkey 181, i.p.	Survived	No fever.
194	„	5 c.c. 20 p.c. suspension, mouse brain, 34th passage, i.p.	„	„
195	„	5 c.c. 20 p.c. suspension, mouse brain, 34th passage, i.p.; 1 c.c. 2 p.c. starch, i.c.	10	Encephalitis.
4	*M. irus*	0·75 c.c. 20 p.c. suspension, mouse brain, 15th passage, i.c.	6	„

i.c. = intracerebral inoculation.
i.p. = intraperitoneal inoculation.

Rhesus monkey 181 was lightly anæsthetized with ether, and 1 c.c. of a 20 p.c. suspension in saline of mouse brain infected with neutrotropic Rift Valley fever virus was instilled intranasally into the nostrils, 0·5 c.c. into each nostril. No symptoms developed till the 5th day after inoculation, when the temperature rose to 104° F. and remained between 104° and 105° till the 8th day, when it fell to 98·8°. Weakness began to develop in the legs on the 7th day after inoculation and rapidly spread to the right arm; the monkey was killed when moribund (Chart 5). Tests were made for the presence of virus in the blood 24 hours after intranasal instillation, on the 5th day, and again at death in the blood and other tissues; before batches of 6 mice were inoculated, but in this instance batches were injected both intracerebrally and intraperitoneally. All the mice dying developed symptoms of nervous involvement. The results are seen in Table III.

Rhesus monkey 195 was inoculated intracerebrally with 1 c.c. of a 2 p.c. suspension of starch in saline, and intraperitoneally with 5 c.c. of a 20 p.c. suspension in saline of mouse brains infected with neurotropic Rift Valley fever after 34 passages in mouse brains; as a control *Rhesus monkey* 194 received a similar intraperitoneal inoculation, but no starch intracerebrally. Monkey 195 showed no symptoms till the 5th day after inoculation, when the temperature rose from 102·4° to 104·2°. The temperature fluctuated between 104° and 105° till the 9th day after inoculation, when it began to sink, final collapse occurring on the 10th day after inoculation, when the animal was killed moribund. Nervous symptoms, weakness in the legs and twitching of the right arm began to develop on the 8th day after inoculation. Monkey 194 showed no febrile reaction except that on the 5th day after inoculation the temperature just reached 104°. No nervous symptoms were seen.

TABLE III.—*Distribution of Neurotropic Rift Valley Fever Virus in the Blood and Tissues of Monkeys as shown by Intracerebral Injection of 6 Mice.*

Number of monkey.	Route of inoculation.	Inoculum.	Virus in— Blood. 1 day after inoculation.	Blood. 3 days after inoculation.	Blood. 5 days after inoculation.	Brain.	Liver.	Spleen.	Adrenal.
176	i.c.	Mouse brain, 10th passage	†9	†4	†4	†3	†4	†3	†3
			S	4	4	3	5	4	3
			S	4	5	4	5	5	3
			S	5	5	4	6	5	4
			S	5	6	4	6	6	4
			S	7	6	4	7	6	5
			1 day after inoculation.	3 days after inoculation.	5 days after inoculation.				
177	i.c.	Mouse brain, 12th passage	†5	†2	†3	†3	S	S	S
			S	2	7	3	S	S	S
			S	3	S	3	S	S	S
			S	3	S	4	S	S	S
			S	4	S	5	S	S	S
			S	7	S	5	S	S	S
			1 day after inoculation.	3 days after inoculation.	6 days after inoculation.				
179	i.c.	Monkey 176 brain	†4	†3	†3	†3	†4	†3	†3
			5	3	3	3	5	5	3
			S	4	4	3	5	5	4
			S	4	4	4	6	6	4
			S	5	5	4	S	6	5
			S	5	5	6	S	S	5
			1 day after inoculation.	6 days after inoculation.	10 days after inoculation.				
180	i.p.	Mouse brain, 20th passage	†6	†13	S				
			7	S	S				
			9	S	S				
			S	S	S				
			S	S	S				
			S	S	S				

| | | |

TABLE III—*continued.*

Number of monkey.	Route of inoculation.	Inoculum.	Virus in— Blood. 1 day after inoculation.	Blood. 6 days after inoculation.	Blood. 10 days after inoculation.	Brain.	Liver.	Spleen.	Adrenal.
195	i.p. + starch i.c.	Mouse brain, 34th passage	†4	†5	S	†3	S	S	†5
			5	5	S	3	S	S	8
			5	S	S	3	S	S	17
			6	S	S	3	S	S	S
			S	S	S	3	S	S	S
			S	S	S	3	S	S	S
			1 day after inoculation.	3 days after inoculation.	6 days after inoculation.				
Macaca irus 4	i.c.	Mouse brain, 15th passage	S	†6	S	†5	†8	†8	†5
			S	7	S	5	15	5	6
			S	8	S	5	S	S	6
			S	S	S	5	S	S	8
			S	S	S	5	S	S	S
			S	S	S	6	S	S	S

i.c. = Intracerebral inoculation. i.p. = intraperitoneal inoculation. † = death; the number following indicates the number of days from inoculation to death. S = survival.

DISTRIBUTION OF VIRUS IN THE TISSUES.

After intraperitoneal inoculation of neurotropic virus in the absence of cerebral trauma, there is a rapid disappearance of virus from the circulating blood-stream. The clearing mechanism of the peripheral blood-stream has been discussed more fully in connection with experiments on mice (Mackenzie, Findlay and Stern, 1936). After intracerebral inoculation, in the earlier passages virus was usually present in the blood-stream at death, but in the later passages, both after intracerebral inoculation and after nasal instillation (monkey 182), there was a tendency for the blood-stream to be free from virus. In monkey 195 that received virus intraperitoneally and starch intracerebrally, virus was not found in the liver or spleen, but was present only in the brain and adrenal. The sciatic nerve in this case also contained virus; for, of 6 mice inoculated intracerebrally, 3 died with encephalitis, in 6, 8 and 11 days after inoculation. Monkey 187 presents certain interesting features. It was inoculated with neurotropic virus intraperitoneally and starch intracerebrally, and showed a temporary infection of the blood-stream. Although encephalitic symptoms were present at death, virus was only found in small amounts in the brain and was absent from all other organs. In view of these findings tests were made in mice to determine whether immune bodies were already present in the blood. It was found that the serum was capable of neutralizing 1000 M.L.D. pantropic Rift Valley fever virus. Similar development of immune bodies in the peripheral blood-stream with death from encephalitis has been recorded in the case of neurotropic yellow fever by Findlay and Clarke (1935). As a result of passage through monkeys there was no tendency for the neurotropic strain to recover its pantropic pathogenicity, for all the mice dying as a result of inoculation with mouse tissues developed encephalitic symptoms.

HISTOLOGICAL CHANGES.

The lesions produced by the neurotropic strain of Rift Valley fever virus in lambs and monkeys differed from those produced by the pantropic strain. The cerebral lesions noted in lambs and monkeys were, however, essentially similar to those produced by the neurotropic virus in rodents, so that only a very brief description is necessary.

Lambs.—The abdominal and thoracic viscera appeared quite normal. The liver showed no fatty infiltration or degeneration and no necrosis of the liver-cells. No intranuclear inclusions were present. In the brain and cord there was very slight involvement of the meninges, which at the base of the brain showed slight infiltration with lymphocytes. In the cerebral cortex there was very little inflammatory change, though here and there a degenerating nerve-cell was seen. In the mid-brain the inflammatory and degenerative changes were more marked, especially in the walls of the third and fourth ventricles, where an occasional blood-vessel showed perivascular infiltration. The Purkinjë cells of the cerebellum were relatively unaffected, only very few cells showing degenerative changes. The pathological changes in the brain of lambs were thus comparatively slight in extent, probably owing to the intense rapidity of the toxic process.

In *monkeys* also there were no lesions in the viscera, the liver being entirely free from areas of focal necrosis. In the central nervous system, however, the changes were more marked than in lambs, though similar in character, consisting of infiltration of the meninges with lymphocytes, perivascular infiltration, degeneration of the ganglion-cells and here and there small areas of focal necrosis. All stages of degeneration were found in the ganglion-cells, and many were undergoing neuronophagia. In certain of these cells acidophilic intranuclear bodies could be seen, similar in character to those described in the brain of mice, and very closely akin to those met with in the brains of monkeys dying with yellow fever encephalitis.

DISCUSSION.

The observations here recorded show that by repeated passage in mouse brains the virus of Rift Valley fever has been rendered increasingly neurotropic, not only for mice, but for other species such as the sheep and monkey. At the same time the pathogenicity of the virus administered by subcutaneous inoculation has been decreased, so that sheep and lambs no longer suffer from a severe necrosis of the liver.

There is thus a possibility that the neurotropic strain may be of value for the purposes of mass immunization of sheep in countries, such as Kenya, where ordinary Rift Valley fever occasionally occurs in epizootic form. The danger of producing encephalitis in persons administering the virus to sheep might, however, be an objection to this procedure. It would seem desirable that, as in the case of yellow fever virus, those handling the neurotropic strain of Rift Valley fever virus should possess immune bodies to the pantropic strain. If immune bodies are present in the blood there is little danger of producing encephalitis, as is shown by the following experiment: Two rhesus monkeys which had acquired immunity to the pantropic strain of Rift Valley fever virus six months previously were anæsthetized with ether, and were each inoculated intranasally with 2 c.c. of a 20 p.c. suspension of mouse brain infected with the neurotropic strain of Rift Valley fever virus. Neither of the monkeys developed encephalitis or showed any significant rise of temperature during the next four weeks.

The production of a neurotropic strain of Rift Valley fever virus indicates its close similarity to yellow fever, although the two diseases yield no cross-immunity, as shown both by experiments *in vivo* and *in vitro*. It is not perhaps so commonly realized that dengue, which clinically is not unlike Rift Valley fever, may also on occasion give rise to encephalitic symptoms. Thus during the epidemic in Greece, Avaritsiotis (1931), Bensis (1931) and others described nervous phenomena in association with attacks of dengue, while Cheney (1935) produced encephalitis in a volunteer inoculated subcutaneously with a virus obtained from a dengue-like fever in California.

Were a suitable experimental animal available, it seems not improbable that a neurotropic strain of dengue could also be easily produced by the technique that has been successful in yellow fever. Unless the similarities displayed by the viruses of yellow fever, Rift Valley fever and dengue are due to evolutionary convergence, it seems possible that they may have originally sprung from some common ancestral form.

SUMMARY.

The neurotropic strain of Rift Valley fever virus produces encephalitis when inoculated intracerebrally in lambs. Subcutaneous inoculation into sheep and lambs does not cause illness.

Immunity against the pantropic strain follows the subcutaneous injection of neurotropic virus in sheep and lambs.

Two species of monkey, *Macaca mulatta*, the rhesus monkey, and *M. irus*, the crab-eating macacque, are highly susceptible to intracerebral inoculation of neurotropic Rift Valley fever virus and die with encephalitis.

With an increase in the number of mouse brain passages there is a greater tendency for the virus, when injected intracerebrally in monkeys, to be found exclusively in nervous tissues.

REFERENCES.

AVARITSIOTIS, E.—(1931) *Ann. Méd.*, **30**, 5.
BENSIS, W.—(1931) *Paris méd.*, **2**, 137.
CHENEY, G.—(1935) *Arch. int. Med.*, **56**, 1067.
DAUBNEY, R., HUDSON, J. R., AND GARNHAM, P. C.—(1931) *J. Path. Bact.*, **34**, 545.
FINDLAY, G. M.—(1931–32) *Trans. R. Soc. trop. Med. Hyg.*, **25**, 229.—(1932–33) *Ibid.*, **26**, 161.
Idem AND CLARKE, L. P.—(1935) *J. Path. Bact.*, **40**, 55.
Idem AND DAUBNEY, R.—(1931) *Lancet*, **2**, 1350.
Idem AND STERN, R. O.—(1935) *J. Path. Bact.*, **41**, 431.
MACKENZIE, R. D., AND FINDLAY, G. M.—(1936) *Lancet*, **1**, 140.
MACKENZIE, R. D., FINDLAY, G. M., AND STERN, R. O.—(1936) *Brit. J. exp. Path.*, **17**, 352.

STUDIES ON NEUROTROPIC RIFT VALLEY FEVER VIRUS: "SPONTANEOUS" ENCEPHALOMYELITIS IN MICE.

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ONE of the phenomena in connection with viruses that still requires explanation is the occurrence on rare occasions of post-febrile encephalitis. Cases of encephalitis have been recorded not only in association with vaccinia, but also in relation to variola, cowpox, varicella, measles, mumps and rubella. Less commonly encephalitic symptoms have been recorded as sequelæ to yellow fever and dengue. Certain viruses concerned, such as that of yellow fever, possess specific neurotropic affinities, since the neurones themselves are affected; others, like vaccinia, attack only the mesodermal tissues of the central nervous system.

Three theories have been suggested to account for the development of post-febrile encephalitis: (1) Infection of the central nervous system with a virus the affinities of which have been increased for nervous tissue; (2) infection of the central nervous system with a non-specific virus stirred into activity by the primary infection; (3) a toxic and degenerative process, primarily, but not immediately, due to infection with a specific virus.

SPONTANEOUS ENCEPHALITIS IN RIFT VALLEY FEVER.

During the past six months, in the course of experiments on Rift Valley fever, two apparently spontaneous cases of encephalitis have been encountered in mice infected with the pantropic strain of Rift Valley fever virus. As the conditions under which these cases of encephalitis arose are accurately known, it is not without interest to place them on record.

Mouse 1 was one of a batch of 6 adult mice inoculated intraperitoneally with a mixture of 0·015 c.c. of Rift Valley fever immune serum and 0·015 c.c. of a suspension of mouse liver infected with Rift Valley fever virus diluted 10^{-2}, containing approximately 750,000 M.L.D. Two of the 6 mice died with ordinary Rift Valley fever 5 and 10 days after inoculation. Twenty-two days after the primary inoculation the 4 surviving mice were again inoculated intraperitoneally with 0·2 c.c. of a suspension of mouse liver infected with pantropic Rift Valley fever virus diluted 10^{-2}, containing approximately 10,000,000 M.L.D.

Three of the mice survived and showed no unusual symptoms. The fourth mouse, 7 days after inoculation, became completely paralysed in the hind legs and was killed for further examination. At the post-mortem the liver was normal in appearance, and histologically it showed none of the lesions characteristic of Rift Valley fever; the central nervous system, on the other hand, presented the typical picture of encephalo-myelitis. Emulsions of the brain and spleen and also serum were inoculated intracerebrally into batches of 6 mice in doses of 0·03 c.c. The results are given in Table I:

TABLE I.

Brain.	Spleen.	Blood.
†3	†3	S
†3	†3	S
†3	†4	S
†3	†4	S
†3	†4	S
S	S	S

S = survival. † = death; the accompanying figure indicates the number of days from inoculation to death.

None of the mice developed nervous symptoms, and in all those that died the liver had the appearances characteristic of pantropic Rift Valley fever. In order to make certain that the virus responsible for the original cerebral symptoms had not become "fixed" for nervous tissue, a second passage was

made with virus from the brains of one of the mice dying three days after intracerebral inoculation. Six mice were inoculated intraperitoneally with 0·2 c.c. of a 20 p.c. suspension of mouse brain, and 6 mice intracerebrally with 0·03 c.c. of the same suspension; all the mice of both batches died 48 hours after inoculation without cerebral symptoms, but with advanced necrosis of the liver.

Mouse 2 was one of a batch of 10 mice inoculated intraperitoneally with 0·2 c.c. of undiluted immune Rift Valley fever serum and 0·2 c.c. of a suspension of mouse liver infected with Rift Valley fever virus diluted 10^{-3}, containing approximately 1,000,000 M.L.D.; all the mice survived this inoculation and 10 days later were injected with viscerotropic virus without serum, the inoculum consisting of 0·2 c.c. of a suspension of infected mouse liver diluted 10^{-2}, containing approximately 10,000,000 M.L.D. Eight of the 10 mice survived this injection, but 2 died, one after 2, the other after 4 days without nervous symptoms, but with typical liver necrosis.

Thirty-five days after the second inoculation the 8 survivors were again inoculated intraperitoneally with 0·2 c.c. of mouse liver infected with viscerotropic Rift Valley fever virus, diluted in saline 10^{-2}. Seven of the mice remained without symptoms; the other mouse, 8 days after inoculation, was found to be rolling over and over sideways and turning in circles. The mouse was killed. The liver appeared normal at post-mortem and histologically showed no change beyond slight fatty infiltration. The brain showed the appearances characteristic of encephalomyelitis. Blood-serum and 20 p.c. emulsions of liver, spleen and brain in saline were inoculated intracerebrally and intraperitoneally into mice in doses of 0·2 c.c. and 0·03 c.c. respectively.

The results are given in Table II.

TABLE II.

Blood.		Brain.		Liver.		Spleen.	
Intra-peritoneal.	Intra-cerebral.	Intra-peritoneal.	Intra-cerebral.	Intra-peritoneal.	Intra-cerebral.	Intra-peritoneal.	Intra cerebral.
S	S	†2	†2	†7	†5	†3	†3
S	S	†2	†2	†9	†5	†3	†3
S	S	†2	†2	S	†11	†3	†4
S	S	†3	†2	S	S	†4	†4
S	S	†3	†3	S	S	†4	†4
S	S	†4	S	S	S	S	†4

S = survival. † = death.

It will be seen that no virus was demonstrated in blood and only a small amount in liver; brain and spleen, however, contained large amounts. None of the mice that died, whether injected with liver, spleen or brain, developed cerebral symptoms, but all had typical necrosis of the liver.

In both mice that developed spontaneous cerebral symptoms following intraperitoneal inoculation there is thus nothing to suggest that the viruses producing the cerebral symptoms were possessed of increased neurotropism; both mice, however, showed evidence of some degree of immunity, since in

neither could virus be demonstrated in the blood. By some means, therefore, in partially immunized mice, virus had gained entrance into the central nervous system, and had there been able to multiply and produce encephalitic lesions.

The evidence thus suggests that the degree of active immunity present in the central nervous system is not necessarily as great as that present in the viscera, and that the mechanism by which the encephalitis is produced in these cases is akin to that experimentally employed by Findlay and Stern (1935) and Mackenzie and Findlay (1936) in demonstrating the essential neurotropism of the pantropic strains of yellow fever virus and Rift Valley fever virus.

INTRACEREBRAL INOCULATION OF PANTROPIC VIRUS IN ACTIVELY IMMUNIZED MICE.

The effects of injecting pantropic Rift Valley fever virus intracerebrally in mice which had withstood intraperitoneal inoculations of the same virus were therefore studied.

Seven mice that had been injected intraperitoneally with pantropic virus and immune serum, and had subsequently received at least two inoculations of pantropic virus, were injected intracerebrally with 0·03 c.c. of serum from a mouse dying with ordinary Rift Valley fever. Six of the mice showed no evidence of infection, but the seventh developed nervous symptoms 5 days after inoculation and died 3 days later. No lesions were found in the liver, but encephalitic changes were present in the brain. An attempt was made to determine the distribution of virus in this mouse by intracerebral inoculation of brain, blood and liver into other mice. No virus could be demonstrated either in the blood, liver or brain. The findings were thus typical of the " infection autosterilisable " of French writers.

However, it appeared not impossible that the absence of virus might be due to the fact that the trauma incidental to intracerebral inoculation had by damaging blood-vessels liberated into the tissues immune bodies contained in the blood-serum. An attempt was therefore made to infect mice intranasally with pantropic Rift Valley fever virus, since by this means actual trauma to the brain is avoided.

TABLE III.

No. of mouse.	Immune mice.	Normal mice.
1	†4 nervous symptoms	†4 no nervous symptoms
2	†6 ,,	†4 ,, ,,
3	†6 ,,	†4 ,, ,,
4	†7 ,,	†4 ,, ,,
5	†8 ,,	†5 ,, ,,
6	†8 ,,	†5 ,, ,,
7	†8 ,,	†5 ,, ,,
8	S	†5 ,, ,,
9	S	†6 ,, ,,
10	S	†6 ,, ,,
11	S	†7 ,, ,,
12	S	S

S = Survival. † = Death.

Twelve mice which had received immune serum and virus intraperitoneally followed by two intraperitoneal inoculations of virus were anæsthetized by ether, and 0·03 c.c. of a suspension in saline, diluted 10^{-2}, of mouse liver infected with pantropic Rift Valley fever virus was instilled intranasally. Twelve control mice were similarly inoculated.

From Table III it will be seen that 7 of the immune mice inoculated intranasally subsequently developed encephalitis and died. At death tests were made for the presence of virus in the blood-serum and in the brain. In all cases the blood failed to show the presence of virus, but virus could occasionally be demonstrated in the brain, especially if the brain-tissue was diluted in saline. The control mice died of ordinary Rift Valley fever.

PRESENCE OF PANTROPIC VIRUS IN THE BRAINS OF MICE AFTER NASAL INSTILLATION, THE MICE HAVING PREVIOUSLY BEEN IMMUNIZED BY INTRAPERITONEAL INOCULATION.

From Table IV it will be seen that in 2 of the "immune" mice no virus could be demonstrated in the brain, even on dilution, though they undoubtedly died with encephalitic symptoms; in 3 mice virus became apparent on dilution; in 2 it was demonstrable without dilution. There is thus a close similarity to the dissociable and non-dissociable mixtures of immune serum and Rift Valley fever virus discussed by Findlay (1936). Failure to demonstrate virus in the brain of an animal that has died from encephalitis by injection of a concentrated emulsion of tissue is thus not absolute proof that virus is absent from the tissues.

TABLE IV.

No. of mouse.	Dilution of brain tissue. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
1	0000	00++	0+++	000+
2	+++0	++++	++++	++++
3	0000	000+	0+++	0+++
4	0000	0000	0000	0000
5	++00	+++0	++00	+000
6	0000	0000	0000	0000
7	0000	00++	00++	000+
Control 1	++++	++++	+++0	++++
,, 2	++++	++++	++++	++++

ANAPHYLACTIC SYMPTOMS IN RELATION TO RIFT VALLEY FEVER.

During the course of these experiments certain phenomena, possibly of an anaphylactic nature, were observed in mice that possessed a high degree of immunity to the intraperitoneal inoculation of pantropic Rift Valley fever, as shown by their tolerance to intraperitoneal inoculations of 0·2 c.c. of infected mouse liver diluted 10^{-2}, as detailed on p. 443. Following on the intracerebral inoculation of 0·03 c.c. of infected mouse liver diluted 10^{-2} 5 weeks after the last intraperitoneal inoculation, there developed in these mice very definite symptoms of sickness within a minute of the inoculation. The mice

showed rapid breathing and roughened fur; they either collapsed altogether, or remained huddled in a corner. These symptoms continued for from 10 to 15 minutes, and then gradually passed off; in no case did the mouse die. It may be recalled that Hindle (1929) reported that certain monkeys which had been hyperimmunized against yellow fever died within a few hours of receiving a further injection of infected monkey liver. The results were apparently not attributable to direct toxicity of the liver, for the inoculation of large doses of infected spleen and normal liver into 2 hyperimmunized monkeys failed to cause any reaction.

In order to determine whether infected mouse liver was the only tissue capable of producing these symptoms in mice, 6 mice immune to intraperitoneal inoculation of pantropic Rift Valley fever virus were given an intracerebral inoculation of 0·03 c.c. of a 20 p.c. suspension of mouse brain in saline. In these mice the symptoms were similar in character but more severe in degree, since 3 of the 6 mice died.

Immediate examination revealed nothing beyond dilatation of the right side of the heart. Intracerebral inoculation of normal mouse liver and brain into immune mice did not give rise to any symptoms of illness. It therefore seems that infected tissues are necessary to produce the symptoms, but, in view of the well-known resistance of mice to anaphylactic shock, some hesitation is felt in ascribing the symptoms to true anaphylaxis. Further experiments on this subject are in progress.

DISCUSSION.

Certain conclusions may be drawn from the cases of spontaneous encephalitis in mice here described and the experiments to which these cases gave rise. In the first place, even when a high degree of immunity is possessed by the viscera, either as a result of passively injected or actively produced immune bodies, the central nervous system may not necessarily possess the same degree of immunity, and if virus reaches this relatively unimmunized tissue, encephalitis may result. The paths by which virus may reach the central nervous system are still uncertain. Either the virus may penetrate the hæmato-encephalitic barrier, or it may be excreted on to the nasal mucosa and thence pass up the olfactory nerve-endings to reach the brain. Francis and Magill (1935) have already found that in man Rift Valley fever virus may be excreted on to the nasal mucosa.

The second conclusion of interest is that the strain of virus giving rise to encephalitis does not possess any increased neurotropism over and above that possessed by the ordinary pantropic virus. This applies also to yellow fever (Findlay and Stern, 1935), and may well be true of many other viruses that, on occasion, may produce encephalitis as a result of gaining access to the central nervous system.

Finally, failure to demonstrate virus in the central nervous system in cases of spontaneous post-febrile encephalitis is not proof that the encephalitis has not been produced by a virus. The virus and the immune bodies present in the central nervous system may have formed a mixture that can only be dissociated by dilution, or, on the other hand, they may have formed a non-dissociable mixture.

In cases of post-vaccinial encephalitis the presence of demyelinization has been regarded as contra-indicating the action of a virus. The exact conditions responsible for demyelinization are at present unknown, but it is doubtful whether the presence of demyelinization necessarily excludes virus activities. Perdrau (1936), for instance, has recently shown that Australian X disease is regularly associated with demyelinization, while Lhermitte and Fribourg-Blanc (1936) have recorded a case in man of subacute encephalo-myelitis following the subcutaneous inoculation of neurotropic yellow fever virus; extensive demyelinization was present *post mortem*. Demyelinization may thus be associated with the action of viruses on the central nervous system.

It seems probable that, as in the spontaneous Rift Valley fever encephalitis here described, so in some other post-febrile forms of encephalitis following virus infections, the nervous symptoms may be explained as due to the involvement of a non-immune nervous system at a time when a visceral immunity has already developed. If the central nervous system becomes immunized at the same time as the viscera, encephalitis will not occur; if there is delay in the development of immunity in nervous tissue, then encephalitis my occur.

SUMMARY.

Two cases of spontaneous encephalitis in mice are recorded following intraperitoneal inoculation of pantropic Rift Valley fever virus.

The viruses isolated from the brains of these two mice did not show enhanced neurotropism.

Both the mice developing spontaneous encephalitis already possessed a high degree of visceral resistance to infection with Rift Valley fever virus.

In mice possessing a high degree of visceral resistance to infection with Rift Valley fever virus, intracerebral inoculation or intranasal instillation of pantropic virus may lead to the development of encephalitis.

In such cases of encephalitis the presence of virus in the brain may be (*a*) readily demonstrated, (*b*) demonstrated only on dilution, (*c*) undemonstrable.

The bearing of these findings on encephalitis following infection with other viruses is briefly discussed.

REFERENCES.

FINDLAY, G. M.—(1936) *Brit. J. exp. Path.*, **17**, 89.
Idem AND STERN, R. O.—(1935) *J. Path. Bact.*, **41**, 431.
FRANCIS, T. (jun.), AND MAGILL, T. P.—(1935) *J. exp. Med.*, **62**, 433.
HINDLE, E.—(1928–29) *Trans. Roy. Soc. trop. Med. Hyg.*, **22**, 405.
LHERMITTE, J., AND FRIBOURG-BLANC.—(1936) *Rev. neurol.*, **65**, 391.
MACKENZIE, R. D., AND FINDLAY, G. M.—(1936) *Lancet*, **1**, 140.
PERDRAU, J. R.—(1936) *J. Path. Bact.*, **42**, 59.

THE TYPHUS GROUP OF DISEASES IN MALAYA. PART VII: THE RELATION OF RURAL TYPHUS TO THE TSUTSUGAMUSHI DISEASE (WITH SPECIAL REFERENCE TO CROSS-IMMUNITY TESTS).

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THE occurrence in Malaya of rural typhus (scrub-typhus) was established by Fletcher and Lesslar (1925); and of the tsutsugamushi disease by Dowden (1915), who described a case which was probably tsutsugamushi, and by Fletcher and Field (1927). The clinical features were described in detail by these workers. Fletcher and Field considered them to be separate diseases. Fletcher, Lesslar and Lewthwaite (1929), reviewing the early cases of the comparatively rare tsutsugamushi disease, reported as follows:

"The tsutsugamushi disease of the East Indies and scrub-typhus both occur in circumscribed areas which are covered with undergrowth, and their symptoms are much alike. There are, however, the following points of distinction: (*a*) The fever of tsutsugamushi does not end abruptly at the end of the second week as it does in scrub-typhus, nor is convalescence so rapid. (*b*) A primary sore and bubo are present in tsutsugamushi, but absent in scrub-typhus. (*c*) The titre of agglutination with *B. proteus*, Kingsbury, is low in the tsutsugamushi disease, but very high in scrub-typhus. In some cases of the tsutsugamushi disease the serum agglutinates *B. proteus*, Kingsbury, in higher dilutions than that of normal persons, but not to titres nearly so high as in scrub-typhus. It is suggested that the two diseases have a similar ætiology and that scrub-typhus, like the tsutsugamushi disease, is carried by trombiculæ."

Felix and Rhodes (1931), however, examining sera from a larger series of tsutsugamushi cases from Japan and Sumatra, showed that high and constant agglutination of the OXK type occurred. These findings were confirmed by Wolff (1931) in Sumatra; and also by the present authors in a series of some twenty cases subsequently observed in Malaya, the agglutination of the OXK type being invariable where repeated samples of serum were available during convalescence. Observation of these cases also showed that the mortality, and the duration of fever and convalescence, were much the same in both diseases. Thus the occurrence of the primary ulcer and bubo in tsutsugamushi remained the sole diagnostic criterion.

Kouwenaar and Wolff (1936) consider that scrub-typhus (*i. e.* rural typhus) is Sumatran mite-fever without the primary ulcer; but that mite-fever and tsutsugamushi are two different diseases.

Anigstein (1933), discussing the causal organism of rural typhus, states:

"As regards the systematic position of the above organism it seems to the writer that the *Rickettsia* of tropical typhus, being distinct from *Rickettsia prowazeki*, is rather related to *R. orientalis* of tsutsugamushi. It would be, however, premature to create of the discovered organism a new *Rickettsia* species, since the close serological relationship between scrub-typhus and tsutsugamushi has been established (Wolff, 1932). In addition to this fact the recent findings of Lewthwaite (*Ann. Rep. I.M.R.* for 1932) pointing to a cross-immunity between the two diseases are very suggestive as regards their identity. If this is found to be the case the question arises, however: Why are the clinical features of tsutsugamushi, especially the characteristic ulcer, so distinct as compared with those of scrub-typhus? The present state of the investigations on this problem does not yet permit one to conclude that the two viruses are identical. In the writer's opinion they represent merely distinct biological variations of a *Rickettsia* species."

To elucidate the relationship between the two diseases, viruses derived from human cases of each of them were established in laboratory animals, and the experimental diseases compared. The findings are reported below, some in brief only, since they have been reported in detail in earlier parts of this series of papers (1936*a*, *b*, *c*, *d*, *e*); a large series of cross-immunity experiments are reported for the first time.

CAUSAL ORGANISM.

Both diseases are caused by *Rickettsia*, which are demonstrable in material from infected guinea-pigs and rabbits. In numbers, morphology, distribution and staining characteristics, the respective *Rickettsia* are identical with one another and with the *Rickettsia orientalis* of Nagayo (1931) (*vide* Parts I and II).

SUSCEPTIBILITY OF LABORATORY ANIMALS.

Guinea-pigs.—These animals are relatively insusceptible to infection with the viruses. It has been noted in Part II of this series that, in the few guinea-pigs that have reacted to tsutsugamushi virus, the reaction was identical in clinical and post-mortem features with that characteristic of experimental rural typhus, as seen in guinea-pigs of the "Seerangayee" strain.

Rabbits.—These are the animals of choice. Infection by the intra-ocular route is secured without undue difficulty; an irididocyclitis results, the evolution of which is identical in all respects in the case of either experimental disease.

Monkeys.—Attempts to infect these animals by the intradermal inoculation of infected material from passage animals of either experimental disease succeeded; the signs of infection were the same in each case, viz. febrile reaction, leucopenia, initial dermal lesion, glandular enlargement, and the development of a positive Weil-Felix reaction (*vide* Parts I and II).

SEROLOGY.

In the sera of rabbits inoculated by the intra-peritoneal or intra-ocular route with the virus of rural typhus or tsutsugamushi, OXK agglutinins developed in approximately 60 p.c. of the animals. The degree of agglutination attained, and the lapse of time between inoculation and the development of

the maximum titre, were approximately the same with both viruses. A gibbon and a macacus monkey were inoculated intradermally with passage virus from a strain of tsutsugamushi, and a gibbon and three macacus monkeys by the same route with passage virus from a strain of rural typhus. In all 6, agglutinins of the OXK type developed in the serum to a significant degree. The titres were higher with the tsutsugamushi strain—a difference that may be regarded merely as an individual variation between two strains of the XK type (*vide* Part V).

CROSS-IMMUNITY.

Experiments have been carried out in the guinea-pig, rabbit, and monkey.

The Guinea-pig.

One strain of rural typhus and two strains of tsutsugamushi were used. They are described in brief below.

Rural typhus.—The " Seerangayee " strain : Of more than a hundred attempts made to establish strains in the guinea-pig from human cases, one only was successful, the " Seerangayee " strain being established ; it has been maintained with unabated virulence for four years.

Tsutsugamushi.—(1) The " Kepong " strain : The initiation of this strain in the guinea-pig was the only success of eleven attempts made with virus from human cases. It could only be maintained for nine generations, and that with great difficulty. (2) The " Raub " strain : This strain was initiated in rabbits four years ago by the intra-ocular inoculation of human virus, and has been maintained in rabbits since then by similar inoculation of infected aqueous humour. Fifteen attempts have been made, with partial success, to transfer the strain to the guinea-pig by the intra-peritoneal inoculation of infected material from the reacting eyes of passage rabbits ; and thereafter to maintain it in the guinea-pig by the intra-peritoneal inoculation of brain material.

Procedure.—At least one month's convalescence was allowed to elapse before testing for immunity. The test inoculum was that always used to infect guinea-pigs to secure passage, viz. a suspension of at least one-sixth of the brain of a passage guinea-pig of the stock " Seerangayee " strain of rural typhus, the route of inoculation being the intraperitoneal. In each experiment reported below, whether in guinea-pig, rabbit or monkey, one or more control animals were inoculated, and reacted typically. Little further reference to controls will be made in the text.

Criterion of immunity.—A guinea-pig was presumed to be immune if no reaction was noted during daily observation for 21 days.

Immunity to Reinoculation of Homologous Virus.

Rural typhus.—Twenty guinea-pigs convalescent after infection with the " Seerangayee " strain were reinoculated with a test inoculum of the same strain. All were immune, even guinea-pigs tested fifteen months after their original infection.

Tsutsugamushi.—Owing to the progressive attenuation of the virulence of tsutsugamushi strains from the first generation of passage in the guinea-pig onward, it was not possible to carry out cross-immunity tests with the homologous virus.

Immunity to Reinoculation of Heterologous Virus.

Series 1: "Seerangayee" and "Kepong" Strains.—From guinea-pigs of the fourth generation of the "Kepong" strain of tsutsugamushi, brain virus was inoculated into 2 convalescent guinea-pigs of the "Seerangayee" strain of rural typhus and into 4 control guinea-pigs. The controls all reacted with fever, but the 2 convalescent guinea-pigs remained afebrile. Conversely, 4 guinea-pigs which had been used for passage of the rapidly attenuating "Kepong" strain, and only 1 of which had reacted with fever, were reinoculated with the virus of the "Seerangayee" strain. That guinea-pig that had previously reacted proved to be immune; of the 3 that had previously failed to react with fever, 1 proved to be immune, 2 reacted typically and died of the infection.

Series 2: "Seerangayee" and "Raub" strains.—Five normal guinea-pigs were inoculated with virus from rabbits of the "Raub" strain of tsutsugamushi. One reacted, 4 failed to react. They were reinoculated with passage virus of the "Seerangayee" strain; all proved to be immune. In the course of an attempt to propagate the "Raub" (rabbit) strain in the guinea-pig, infection of 2 guinea-pigs of a second generation was secured, a febrile reaction occurring. They were reinoculated with test virus of the "Seerangayee" strain, and proved to be immune.

Comment.

Thus, in all 6 guinea-pigs (2 of the rural typhus strain, 4 of the tsutsugamushi strains) in which the primary infection had caused a febrile reaction, test inoculation with the heterologous virus showed complete immunity to have resulted. In 7 guinea-pigs that had failed to show febrile reaction following inoculation with either the "Kepong" or the "Raub" strains of tsutsugamushi, reinoculation with test passage virus of rural typhus was made. Five proved to be immune; they were guinea-pigs from the first generation of attempted passage. The other 2 reacted; they were from the fourth generation, and their non-immunity was probably accounted for by the progressive attenuation of the tsutsugamushi virus that is the rule in the guinea-pig.

Thus, between strains of rural typhus and tsutsugamushi in the guinea-pig there is reciprocal cross-immunity.

The Rabbit.

Rural typhus.—Five strains of rural typhus were used; four had been isolated in the rabbit by the intra-ocular inoculation of blood from 4 human cases of the disease; the fifth was indirectly of human origin, having been obtained by the intra-ocular inoculation of heart blood-virus from a reacting guinea-pig of the "Seerangayee" strain of rural typhus, itself derived from a

human case. Maintenance of the strains in rabbits by intra-ocular inoculation of infected aqueous humour afforded little difficulty (*vide* Part I).

Tsutsugamushi.—Two strains were used, isolated in the rabbit by intra-ocular inoculation of blood from two human cases of this disease, and maintained by similar inoculations of infected aqueous humour (*vide* Part II).

Procedure.—At least one month's convalescence was allowed to elapse before testing for immunity. The test inoculum was infected aqueous humour injected into the unused eye. One or more control rabbits were inoculated in every case.

Criteria of immunity.—The criteria relied on were two, the ocular reaction (iridocyclitis) and the development of a positive Weil-Felix reaction. In by far the larger series the former criterion was applied.

Immunity as Judged by Ocular Reaction.

A rabbit was presumed to be immune if no ocular reaction was noted by daily observation for 35 days.

Immunity to reinoculation of homologous virus : Rural typhus.—I. In the case of each of 4 of the 5 strains, rabbits convalescent after the ocular reaction were reinoculated into the unused eye with the virus of the particular strain that had caused the original reaction ; and all proved to be immune. The immunity was shown to be of at least twelve months' duration. Twenty-six such experiments were made. In one or two instances insignificant and transitory signs appeared, barely perceptible and of two to three day's duration only ; such fleeting reactions contrasted strongly with the intensity and duration of the reaction in a passage animal.

II. The 5 strains of rural typhus were compared one with another. Seventeen such experiments were made. Eight of these comprised cross-immunity tests between the 4 strains of direct human origin (*vide supra*), and in all no traces of reaction were observed. The other 9 comprised cross-immunity tests between two strains of direct human origin, on the one hand, and the strain of indirect human origin, *i. e.* that isolated in rabbits from the strain of rural typhus in guinea-pigs, on the other hand. Convalescent rabbits of the strains of indirect human origin showed no trace of reaction to inoculation of the virus of the direct human strains. The results of the converse experiments concurred, though the transient and negligible reaction occurred that was noted above as occasional in the reinoculation of convalescents of individual strains by the homologous virus, and was here almost certainly attributable to the greater virulence of the strain derived from the very virulent guinea-pig strain of rural typhus.

Immunity to reinoculation of homologous virus : Tsutsugamushi.—I. In the case of the " Raub " and " Kepong " strains of tsutsugamushi, convalescent rabbits of each strain were reinoculated with their homologous virus ; 10 such experiments were made, and no trace of reaction occurred.

II. Cross-immunity reactions between the above two strains of tsutsugamushi were carried out. Four convalescent rabbits of the " Raub " strain showed no trace of reaction following reinoculation with virus of the " Kepong " strain. Conversely, 5 convalescent rabbits of the " Kepong " strain proved

to be immune to the virus of the " Raub " strain ; though in 4 of these 5 there occurred the same transient and barely perceptible reaction noted above, due undoubtedly to the greater virulence of the more firmly fixed " Raub " strain, which had at that time been passed through 78 generations as compared with the corresponding 9 generations of the " Kepong " strain.

Immunity to reinoculation of heterologous virus.—The strains compared thus were the " Raub " strain of tsutsugamushi on the one hand, and 4 strains of rural typhus on the other hand.

At the time of the isolation of the second strain (" Kepong ") of tsutsugamushi all the strains in rabbits of rural typhus had been discontinued, to effect economy in rabbits ; and since complete cross-immunity has been demonstrated between the " Raub " and the " Kepong " strains of tsutsugamushi, it was not considered necessary to establish new strains of rural typhus in rabbits in order to duplicate the cross-immunity experiments reported below between the " Raub " strain and the strains of rural typhus.

Seven convalescent rabbits of the tsutsugamushi strain were reinoculated into the unused eye with two strains of rural typhus ; one group of 5 with the virus of the strain of indirect human origin, and another group of 2 with the virus of a strain of direct human origin. Of the first group 4 showed no trace of reaction, and 1 merely a negligible fleeting reaction, the late appearance of which on the 20th day of observation was in marked contrast to the intense reaction in the control rabbit, which reached its zenith on the 7th day. Of the second group, both rabbits showed no trace of reaction. In the converse experiments, 16 convalescent rabbits of the rural typhus strains were reinoculated into the unused eye with virus of the tsutsugamushi strain. One group of 9 were convalescents of three strains of direct human origin, and of them, 7 showed no trace of reaction, while 2 showed a barely perceptible reaction of respectively 1 day's and 2 days' duration only. The other group of 7 were convalescents of the strain of indirect human origin, and in them no trace of reaction occurred.

In a further series of cross-immunity experiments, use was made of rabbits that had been inoculated intraperitoneally with the " Seerangayee " strain of rural typhus. From time to time brain, or other tissue virus, was inoculated intraperitoneally into rabbits, in order to confirm by the result of the Weil-Felix reaction that the signs of reaction in the passage guinea-pigs were still those of typhus, and were not due to one or other of the contaminants that in this animal may simulate typhus in febrile reaction and post-mortem appearances, and supplant the typhus virus. Five such rabbits were used. Four of them had been inoculated intraperitoneally one month previously, and their sera had subsequently developed agglutinins (" O " type) to the " XK " strains of *Proteus* to significant titres, viz. in 2 cases to titres of 1/140 on the 12th and 14th days respectively, and in 2 cases to lower titres during the second and third weeks ; the titres in all 4 had shown the waxing and waning curve of agglutination characteristic of typhus infection. The fifth rabbit had been inoculated intraperitoneally six months previously, and had yielded similarly diagnostic agglutination titres. All 5 were reinoculated intra-ocularly with infected aqueous humour from passage rabbits of the tsutsugamushi strain. In the rabbit in which six months had elapsed since the original (intraperitoneal)

inoculation of rural typhus virus, there ensued a delayed, mild, and brief ocular reaction. In the 4 rabbits in which one month only had elapsed since the original inoculation, no trace of ocular reaction occurred.

Immunity as Judged by the Weil-Felix Reaction.

Weil and Felix (1921) showed that, in the case of louse-borne typhus of East-European origin, when rabbits that have acquired an immunity to typhus are reinoculated with tissue suspension from animals infected with typhus, the X19 agglutinins produced as a result of the primary infection are not re-stimulated. Felix (1933) pointed out that this test for re-stimulation of agglutinins could be used as a test for cross-immunity with two strains of typhus virus, and be of similar significance to that ascribed to the usual experiments for cross-protection in the guinea-pig ; and had been so used by Kuczynski (1927) in rats and Munter (1928) in rabbits in an analysis of the antigenic properties of the viruses of European louse-borne typhus and Rocky Mountain spotted fever. Davis (1935) has used the test in a comparative study of the antigenic structure of the viruses of spotted fever, Sao Paulo typhus, and *fièvre boutonneuse*. This serological test was applied by us in the case of the viruses of rural typhus and tsutsugamushi. A rabbit was presumed to be immune if no significant rise of agglutinins occurred during 60 days following inoculation. Only after the titres produced by the primary infection had fallen to normal limits was reinoculation made.

We have reported in Part V of this series of papers that in the sera of rabbits inoculated with the virus of rural typhus, urban typhus, or tsutsugamushi, the respective agglutinins for *Proteus X* strains develop to significant titres in no more than 60 p.c., whether the route of inoculation be the intraperitoneal or the intra-ocular. Thus in such a rabbit, having reacted serologically once, failure to develop agglutinins in the serum on reinoculation with virus of XK or X19 type cannot always be regarded as proof of the presence of an immunity conferred by the initial inoculation ; the criterion of the re-stimulation of agglutinins can be utilized in these diseases only when consistent findings are obtained in a considerable number of rabbits.

Eighteen rabbits that had developed a positive Weil-Felix reaction following inoculation of the virus of rural typhus or tsutsugamushi (both XK type viruses) were reinoculated, some with the homologous virus, others with the heterologous virus, some intra-ocularly, others intraperitoneally. In 17 no significant rise of agglutinins occurred ; in the 18th, OXK agglutinins rose to a degree slightly higher than the initial one.

Immunity as Judged by the Dermal Lesion.

In the course of a series of experimental infections by the intradermal inoculation of virus (*vide* Parts I and II), 6 rabbits that had previously reacted to intra-ocular inoculation of tsutsugamushi virus and 1 to rural typhus virus were reinoculated on separate occasions intradermally with rural typhus virus. In none did a dermal lesion develop ; whereas typical dermal lesions developed in control rabbits.

Duration of Immunity.

In the case of both the rural typhus and the tsutsugamushi strains in the rabbit, the immunity acquired lasts for at least one year, whether the test inoculum be homologous or heterologous virus.

Comment.

The above results in the rabbit show that between strains of rural typhus and tsutsugamushi in this animal there is complete cross-immunity, whether the route of inculation be intra-ocular, intraperitoneal or intradermal, whether the criterion of reinfection be the development of ocular reaction, positive Weil-Felix reaction, or dermal lesions.

The Monkey.

A macacus monkey, having previously reacted to an intradermal inoculation of rural typhus virus with febrile reaction, dermal lesions, leucopenia and the development of a positive Weil-Felix reaction, was reinoculated intradermally with the homologous virus, along with a control macacus. No trace of any of the above criteria of infection occurred in the reinoculated monkey, whereas the control monkey reacted typically, all four criteria being present.

A gibbon, having previously reacted typically to an intradermal injection of tsutsugamushi virus, was reinoculated intradermally with the virus of rural typhus, together with a control macacus. No reaction other than a mild leucopenia occurred in the reinoculated animal; whereas in the control all four criteria of infection developed.

Summary of Cross-immunity Experiments.

The results of the foregoing experiments in the guinea-pig, rabbit and monkey are in entire accord; and show that cross-immunity between the viruses of rural typhus and tsutsugamushi in these animals is complete.

DISCUSSION.

It has been reported earlier in this paper, and in previous papers of this series, that rural typhus and tsutsugamushi are identical in all known features of ætiology, epidemiology, serology and experimental infections of laboratory animals; and that the clinical syndrome in man is also identical, save for the occurrence of the primary ulcer and bubo in tsutsugamushi. The cross-immunity demonstrated to exist by the comprehensive series of experiments described above constitutes a further addition to the evidence for the identity of the two viruses. The sum of this evidence is sufficiently cogent to raise the question as to how sharply rural typhus and tsutsugamushi can be differentiated as entities by one clinical criterion, viz. the ulcer and its attendant bubo. May it not be that they are one and the same disease in which the dermal lesion varies in the duration and degree of its evolution, being conspicuous, necrotic and persistent in some cases, in others being

inconspicuous, papular only, and fleeting, often escaping recognition by completion of its evolution prior to the onset of other symptoms and signs? Schüffner suggested this possibility many years ago in the case of tsutsugamushi in Sumatra (*vide infra*). We consider that this is the correct explanation of the presence or absence of the dermal lesion; that correlation of the above data with the additional evidence set forth below leads to this conclusion.

A Human Infection Apparently Intermediate Clinically between Rural Typhus and Tsutsugamushi.

A European suffering from a fever of the typhus group was seen in consultation by one of us. Seven days before the onset of illness he had spent the day out shooting amongst lallang on an estate known to be an endemic area of both diseases. The typical syndrome of the tropical typhus-tsutsugamushi group of fevers was present, the blood agglutinated the "OXK" strain of *Proteus* to a titre of 1 : 680 on the 11th day, defervescence by lysis was completed on the 15th day; but delirium was progressive, and death occurred from cardiac failure on the 19th day. During the first few days of illness there was present in the posterior triangle of the neck a tiny red papule, abraded at its apex. The march of the lymphatic involvement of the glands of the posterior triangle, anterior triangle, axilla and groin of the same side, axilla and groin of the opposite side could be clearly followed as the illness progressed, and left no doubt that the papule marked the portal of entry of the virus. Yet, by the eighth day of the disease, early in the development of the rash, the small ulcerated papule had entirely disappeared—an event that was in marked contradistinction to the development of the initial lesion in previous cases of tsutsugamushi reported in this country, the persistence of the ulcer and its black necrotic centre at least until the end of the third week having been the rule even in the mildest case. So insignificant and ephemeral was this ulcer that it would almost certainly have been overlooked on the darker skin of an Asiatic patient.

It would appear that this case, judged by the only adequate clinical criterion, occupied an intermediate position between the two diseases.

The Ulcer in Experimental Animals.

The experimental infections of monkeys and rabbits with the virus of rural typhus or tsutsugamushi by the intradermal route have furnished confirmatory evidence of the wide variation that may occur in degree of dermal reaction at the site of inoculation. The inocula used were from two sources—the eye of an infected rabbit and the peritoneal cavity of an infected guinea-pig. From the eye, either aqueous humour or scrapings from the endothelium covering Descemet's membrane were used; in the former *Rickettsia* are scanty, in the latter abundant. From the peritoneal cavity either ascitic fluid or the fibrinous deposit from the surface of the spleen was used; *Rickettsia* are much more plentiful in the latter than the former. It was frequently demonstrated, usually in the same rabbit or monkey, that the lesions caused by scrapings of the endothelium from Descemet's membrane and by fibrin from the surface of the guinea-pig spleen were of longer duration and greater severity than

those caused by the other two inocula ; they progressed beyond the papular stage to that of necrotic ulceration much more often. Thus ulcer-formation is determined in part by the degree of concentration of virus in the inoculum.

It does not necessarily follow, however, that the development of an ulcer, partially dependent though it may be on a great concentration of virus, is accompanied by an illness more severe than that which occurs in the absence of an ulcer. In experimental rural typhus in the guinea-pig we have found that the inoculation by the intraperitoneal route of minimal infective doses of virus does not appreciably diminish the high death-rate as compared with that resulting from inoculation of doses 100,000 times more concentrated, although it greatly prolongs the incubation period. In fact, in man, in cases recorded in Malaya, there has been no appreciable difference in the death-rates due to the two diseases.

Unequal Incidence of Ulcer in European and Asiatic.

The occurrence of the two diseases within the labour force of an estate and the relative incidence of cases with ulcer and without ulcer (*i. e.* of tsutsugamushi and rural typhus respectively) in European and Asiatic patients calls for comment. Fletcher, Lesslar and Lewthwaite (1928) report human infections or rural typhus and tsutsugamushi contracted in the same endemic area of an estate. Wolff and Kouwenaar (1936) report that Sumatran mite-fever (in which an initial ulcer occurs) and scrub-typhus were diagnosed amongst the same population group of estate labourers. With regard to racial incidence, in the present authors' experience there have been, amongst Europeans, 10 cases of tsutsugamushi for 1 case of rural typhus ; whereas, amongst Asiatics, for 1 case of tsutsugamushi there have been at least 40 cases of rural typhus. The record of cases on a certain estate on which the diseases have been endemic for the past ten years illustrates well the contrasting incidence. Of 6 Europeans that comprise the management, 4 have been infected, in each case the ulcer being present, and the disease, therefore, classified as tsutsugamushi. Among the South Indian labour force, supervised in the field by these Europeans, more than 200 have been infected ; and in all but one no ulcer could be found, the diagnosis being, therefore, that of rural typhus. It would be almost inconceivable that two viruses, closely akin, yet separate entities (as rural typhus and tsutsugamushi have hitherto been regarded), should show such a sharp racial discrimination between European and Asiatic working together in the same areas. There can be no doubt that one and the same virus causes both clinical syndromes. Such is the inference to be drawn from consideration of the infections on this estate, and from the " intermediate " human case cited above, taken in conjunction with the identity of features of the experimental infections in laboratory animals, in particular the results of cross-immunity experiments and intradermal inoculations. It may be recalled that Schüffner (quoted by Wolff, 1931), in an early description of tsutsugamushi in Sumatra, recorded that, having seen the primary ulcer in all his European cases, he considered that its absence in some cases amongst native patients might be due to the fact that the ulcer was healed at the time of admission to hospital.

It must be admitted that the disparity in the incidence of the ulcer according to race may be due to some small extent to the difficulty on the part of the physician of recognizing an inconspicuous dermal lesion in the dark skin of the South Indian ; but this does not wholly account for the disparity. We think that it is in part bound up with the greater sensitivity of the European to mild injury of the skin. The Asiatic of the labouring class wears very scanty clothing while working ; fully 80 p.c. of the cases without ulcer have been dark-skinned South Indian labourers. Constant exposure causes them to be less sensitive to abrasions and bites than are Europeans. These latter, unaccustomed to such exposure, since they wear more clothing, are more sensitive to such mild injury to the skin surface, and more prone to scratch the site of an insect bite, and thereby to enlarge it, and to introduce secondary infection, and perhaps thus to determine the progression of the lesion to the stage of necrotic ulceration.

It may be that in the natural infection of man there are other accessory factors, in addition to the concentration of virus inoculated, that determine the development of an ulcer. One such may be the action at the site of the bite of tissue debilitants, causing localized necrosis ; such may be secondarily infecting organisms introduced into the punctured wound by the mouth parts of the vector, or by scratching or other agency. Again, slight trauma of the wound, due to scratching or the friction of clothes, may be a contributory factor.

Another experimental finding by the authors, that bears on the problem of the dermal lesion, was that no lesion developed at the site of subcutaneous inoculation of the virus of rural typhus. Some authors, including ourselves, have hitherto sought to account for the uncertain incidence of an initial ulcer in diseases due to a virus of the XK type by suggesting that one insect vector with a short proboscis may inoculate a virus intradermally, and another insect vector with a longer proboscis may penetrate the dermis and deposit the same virus subcutaneously. In the former case an ulcer would develop at the site of inoculation ; in the latter case no lesion would develop. Kouwenaar and Wolff consider that scrub-typhus is Sumatran mite-fever without the primary ulcer ; and refer to the possibility that ticks may transmit the virus, and, having a longer proboscis, may inject the virus subcutaneously, so that no ulcer results. In our view to postulate two insect vectors and two routes of inoculation is no longer necessary ; the above conception, based on clinical experience and experimental infections, of gradations of tissue damage that range from an ephemeral insignificant papule to a persistent well-defined necrotic ulcer, following intradermal inoculation, is adequate to explain the presence or absence of a skin-lesion at the time when the patient first comes under the care of the physician.

The Bubo.

One of us (Lewthwaite, 1930), in a description of the clinical syndrome of rural typhus in a series of 164 human cases, reported that a symmetrical enlargement of the lymphatic glands of the axilla and groin was present in 40 p.c. ; that it was rarely very pronounced ; and that it was often accompanied by tenderness. Anigstein, in a similar series of 90 cases, reported similar

findings in 55 p.c. In tsutsugamushi, however, the group of lymphatic glands draining the site of the ulcer is often much more prominent than are the other groups of glands, and occasionally very tender; but it is to be stressed that much variation in the size and tenderness of this bubo is seen, and that in a considerable number of cases with ulcer there is no bubo. The incidence and extent of the bubo are so variable that it cannot be regarded as a criterion between two clinical types. Above was described an "intermediate" human case in which the progressive involvement of the groups of lymphatic glands could be followed, until all were involved. It is probable that, in cases in which the initial lesion has been inconspicuous and fleeting, the same progressive involvement of glands has proceeded, but has been proportionately inconspicuous and fleeting; so that, at the time that the case comes under medical care, no one group of glands predominates.

The Ulcer in Other Typhus-like Fevers.

In other of the typhus-like fevers the same occasional and apparently fortuitous appearance of an ulcer of the skin is found. In the case of the tsutsugamushi disease of Formosa, Morishita, in a personal communication in 1934, informed us that the initial ulcer is not constantly present. Kawamura *et al.* (1935) reported similarly. Such cases without the ulcer would, in Malaya, be classified as rural typhus, but in Formosa are retained within the tsutsugamushi group.

Lépine, in a discussion that followed a paper by us (1936*f*), reported that in *fièvre boutonneuse* "the 'tache noire', which may be compared with the ulcer of the tsutsugamushi disease, may be present or absent; yet it is always the same disease, namely, *fièvre boutonneuse*. The presence or absence of the 'tache noire' or ulcer may be due to the quantity of virus deposited at the site of inoculation".

Megaw, during the same discussion, reported that in a case of tick typhus in India he had seen a local ulcer and bubo; that the ulcer had developed at the site at which the tick was found; but that in the great majority of cases of tick typhus in India no local ulcer and bubo are found.

Again, Unwin (1935), in a recent report from Australia of a series of 1500 cases of "Endemic Tropical Typhus" (Coastal Fever) seen by him in North Queensland, records the following observation: "On close examination of many cases I have been able in several instances to find definite signs of an insect-bite, with its black central eschar and surrounding red areola".

The uncertain incidence of the initial ulcer is thus a clinical feature that is not peculiar to the rural typhus-tsutsugamushi group of fevers in Malaya.

SUMMARY.

1. Cross-immunity tests between strains of rural typhus and tsutsugamushi in the guinea-pig, rabbit and monkey were made. Complete cross-immunity between the strains was demonstrated.

2. The problem of the absence of a primary ulcer in rural typhus and its presence in tsutsugamushi is discussed. Experimental findings are recorded;

from consideration of these and certain clinical and epidemiological observations, the conclusion is drawn that one and the same virus may cause gradations of dermal lesion that vary greatly in extent and duration.

3. Correlation of the results of cross-immunity tests and experimental infections with clinical, ætiological, epidemiological and serological findings indicates that the two diseases are identical. Rural typhus is not a disease *sui generis*, and the term should be discarded, the older designation, " tsutsugamushi disease ", being retained.

REFERENCES.

ANIGSTEIN, L.—(1933) " Studies on Tropical Typhus," *Stud. Inst. med. Res. F.M.S.*

DAVIS, G. E.—(1935) *Publ. Hlth. Rep. Wash.*, **50**, 404.

DOWDEN, R.—(1915) *Ind. med. Gaz.*, **50**, 208.

FELIX, A.—(1933) *Trans. R. Soc. trop. Med. Hyg.*, **26**, 365.

Idem AND RHODES, M.—(1931) *J. Hyg.*, Camb., **31**, 225.

FLETCHER, W., AND FIELD, J. W.—(1927) *Bull. Inst. med. Res. F.M.S.*, No. 1 of 1927.

Idem AND LESSLAR, J. E.—(1925) *Ibid.*, No. 2 of 1925.

Idem LESSLAR, J. E., AND LEWTHWAITE, R.—(1928) *Trans. R. Soc. trop. Med. Hyg.*, **22**, 161.—(1929) *Ibid.*, **23**, 57.

KAWAMURA, R., IMAGAWA, Y., AND ITO, T.—(1935) *Kitasato Arch. exp. Med.*, **12**, 26.

KOUWENAAR, W., AND WOLFF, J. W.—(1936) *Zbl. Bakt.*, 1 Abt., Orig., **135**, 427.

KUCZYNSKI, M. H.—(1927) ' Die Erreger des Fleck- u. Felsenfiebers.' Berlin : J. Springer.

LÉPINE, P.—(1936) *Trans. R. Soc. trop. Med. Hyg.*, **29**, 577.

LEWTHWAITE, R.—(1930) *Bull. Inst. med. Res. F.M.S.*, No. 1 of 1930.

Idem AND SAVOOR, S. R.—(1932) *Rep. Inst. med. Res. F.M.S.*—(1936*a*) *Brit. J. exp. Path.*, **17**, 1.—(1936*b*) *Ibid.*, **17**, 23.—(1936*c*) *Ibid.*, **17**, 208.—(1936*d*) *Ibid.*, **17**, 214.—(1936*f*) *Trans. R. Soc. trop. Med. Hyg.*, **29**, 577.

Idem, HODGKIN, E. P., AND SAVOOR, S. R.—(1936*e*) *Brit. J. exp. Path.*, **17**, 309.

MEGAW, J. W. D.—(1936) *Trans. R. Soc. trop. Med. Hyg.*, **29**, 579.

MUNTER, H.—(1928) *Z. Hyg. InfektKr.*, **109**, 124.

NAGAYO, M., MIYAGAWA, Y., MITAMURA, T., *et al.* (1931) *Jap. J. exp. Med.*, **9**, 87.

UNWIN, M. L.—(1935) *Med. J. Australia*, **2**, 303.

WEIL, E., AND FELIX, A.—(1921) *Z. ImmunForsch.*, **31**, 457.

WOLFF, J. W.—(1931) *J. Hyg.*, Camb., **31**, 352.—(1932) *Geneesk. Tijdschr. Nederl.-Indië*, **72**, 896.

Idem AND KOUWENAAR, W.—(1936) *Ibid.*, **76**, 272.

THE TYPHUS GROUP OF DISEASES IN MALAYA. PART VIII: THE RELATION OF THE TSUTSUGAMUSHI DISEASE (INCLUDING RURAL TYPHUS) TO URBAN TYPHUS. PART IX: THE RELATION OF THE TSUTSUGAMUSHI DISEASE (INCLUDING RURAL TYPHUS) AND URBAN TYPHUS TO ROCKY MOUNTAIN SPOTTED FEVER. (WITH SPECIAL REFERENCE TO CROSS-IMMUNITY TESTS.)

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PART VIII: THE RELATION OF THE TSUTSUGAMUSHI DISEASE* (INCLUDING RURAL TYPHUS) TO URBAN TYPHUS.

IT has been shown earlier that the causal organisms of both diseases are *Rickettsia*, but that they show certain differences. Thus, those of tsutsugamushi are morphologically indistinguishable from *R. orientalis*; those of urban typhus from *R. prowazeki*. In guinea-pigs and rabbits infected with the respective laboratory strains the former organisms are plentiful, the latter scanty.

In epidemiology the diseases differ widely. The tsutsugamushi cases have all been connected by work, domicile or recreation with the countryside; amongst the cases of urban typhus, on the other hand, indoor workers have largely preponderated, many having been employed in handling food and grain.

The principal vector of tsutsugamushi in Malaya is probably a larval mite; the cumulative evidence obtained by a succession of workers strongly suggests that *Trombicula akamushi* and *Trombicula deliensis*, probably forms of the same species of trombidiid mite, will prove to transmit the virus. The principal vector of urban typhus, on the other hand, is almost certainly the rat flea (*Xenopsylla cheopis*); experimental transmission of the virus from rat to rat by this flea was thrice demonstrated, and multiplication of the causal *Rickettsia* within the flea shown to occur (*vide* Part VI).

The descriptions of Fletcher and his co-workers (1925–6–7–9) show that tsutsugamushi and urban typhus in man are difficult to distinguish on clinical grounds, unless the ulcer of tsutsugamushi be present. In urban typhus no initial lesion has ever been recorded. The incidence and mortality of urban typhus are less than those of tsutsugamushi.

* In accordance with the conclusion stated in the preceding paper, strains hitherto designated "rural typhus" are now designated "tsutsugamushi disease".

SUSCEPTIBILITY OF LABORATORY ANIMALS.

The Guinea-pig.

Tsutsugamushi : The guinea-pig relatively is very insusceptible to infection with strains of this disease. Some 250 guinea-pigs have been inoculated with blood drawn from 100 or more patients early in the disease by the authors and by Anigstein (1933) ; only very rarely could a strain be maintained for a few generations, and once only beyond the twelfth generation, and that by recourse to the use of vitamin-deficient diet. The characteristics of the experimental infection with this strain, the " Seerangayee " strain, have been febrile reaction and constant post-mortem signs, viz. ascites, enlargement of the spleen, and the presence of abundant *Rickettsia* in the ascitic fluid and the fibrinous deposit on the surface of the spleen.

Urban typhus : The first attempt to infect guinea-pigs with the virus (human blood) succeeded, and maintenance of the strain (the " Manickam " strain) has been easy. The signs of the infection differ greatly from those in tsutsugamushi. Fever is accompanied by redness, swelling and tenderness of the scrotum in the majority of male guinea-pigs. At post-mortem examination enlargement of the spleen is found, but ascites never. *Rickettsia* are present in the tunica exudate and splenic fibrin, but are scarce.

The Rabbit.

Tsutsugamushi : Initiation of strains by the intra-ocular inoculation of blood drawn early in the disease from 15 patients (with or without initial ulcer) succeeded in 6 instances. Transference of the " Seerangayee " strain from guinea-pig to rabbit was effected several times by intra-ocular inoculation of infected heart blood. Maintenance of these strains by the inoculation of infected aqueous humour into the eye of a normal rabbit was easy ; once the third passage was secured, failure of a rabbit to react was less than 1 p.c. One strain has exceeded 100 generations with unabated virulence. The signs of infection were an intense iridocyclitis in the inoculated eye, the development in the serum of agglutinins for the OXK strains of *Proteus* in 60 p.c. of cases, and the presence of abundant *Rickettsia* in scrapings from the endothelium covering Descemet's membrane. Intra-cutaneous inoculation of virus caused the development of a dermal lesion that usually progressed from papule to necrotic ulcer.

Urban typhus : Rabbits were infected with the virus of urban typhus by intra-ocular injection. But the virus could not be maintained thus, dying out in the third generation. The iridocyclitis was shorter and milder than that evoked by the tsutsugamushi virus. *Rickettsia* were present in scrapings from infected eyes, but were very scanty. The Weil-Felix reaction was positive in 52 p.c. of rabbits thus inoculated ; the agglutinins were for the OX19 strains of *Proteus*. No lesion developed at the site of intra-cutaneous inoculation.

The Monkey.

Gibbons and monkeys were infected by the intra-dermal injection of either virus. Of the signs of infection, fever and leucopenia were alike in both

experimental diseases; two notable and consistent differences were, firstly, that at the site of inoculation a dermal lesion developed in tsutsugamushi, but not in urban typhus, and secondly, that the type of agglutinins that developed in the serum differed.

SEROLOGY.

In human infections and in experimental infections of rabbit and monkey, agglutinins develop in the serum for the OXK strain of *Proteus* in the case of tsutsugamushi, and for the OX19 strain in the case of urban typhus.

In Part V instances were given of dual agglutination, and of changes that occurred in the agglutinogenic properties of two viruses during their maintenance in laboratory animals. Such variations from normal have been extremely rare.

CROSS-IMMUNITY.

Experiments were carried out in the guinea-pig, rabbit and monkey. The procedure and the criteria of immunity were, *mutatis mutandis*, those described in Part VII. Control animals were inoculated in every case, and reacted typically.

The Guinea-pig.

The strains used were the " Manickam " strain of urban typhus and the " Seerangayee " strain of tsutsugamushi.

Immunity to reinoculation of homologous virus.—Tsutsugamushi: As reported earlier (Part VII)—under its former designation of rural typhus—guinea-pigs that recover from an infection with the " Seerangayee " strain develop an immunity that lasts for at least fifteen months.

Urban typhus: Thirty guinea-pigs that had recovered from infection with the " Manickam " strain were reinoculated after intervals of weeks or months with a test inoculum of the same strain. All proved to be immune.

Immunity to reinoculation of heterologous virus.—Six guinea-pigs that had recovered from infection with the " Seerangayee " strain of tsutsugamushi were reinoculated with passage virus of the " Manickam " strain. All reacted typically. Conversely, 22 guinea-pigs that were immune to infection with the " Manickam " strain of urban typhus were reinoculated with the virus of the " Seerangayee " strain. All reacted typically; the death-rate was that of passage animals. Thus there was complete lack of cross-immunity between the two strains in guinea-pigs.

The Rabbit.

The tsutsugamushi strains used were the " Seerangayee " strain, transferred from guinea-pig to rabbit for the purpose, and the " Raub " strain; both were of human origin, and were maintained by the method of intra-ocular inoculation. The urban typhus strain used was the " Manickam " strain, also transferred from guinea-pig to rabbit for the purpose of these tests.

Immunity as Judged by Ocular Reaction.

Immunity to reinoculation of homologous virus.—Tsutsugamushi: It has been recorded earlier (*vide* Part VII) that convalescent rabbits of strains of this disease are immune to reinfection with the homologous virus.

Urban typhus : Three convalescent rabbits of this strain were reinoculated with the same strain, and proved immune.

Immunity to reinoculation of heterologous virus.—Of the tsutsugamushi strains, 4 convalescent rabbits of the "Seerangayee" strain and 4 of the "Raub" strain were inoculated with virus of "Manickam" strain of urban typhus. All 8 reacted characteristically. Conversely, of the urban typhus strain, 2 convalescent rabbits were inoculated with the virus of the "Seerangayee" strain, and 3 with the virus of the "Raub" strain. All 5 reacted characteristically.

Immunity as Judged by Weil-Felix Reaction.

(In the experiments reported below, reinoculation was done only after the titre of the agglutinins resulting from the first inoculation had fallen to normal.)

It was reported in Part VII that 18 rabbits which, following intra-ocular inoculation of the virus of tsutsugamushi, had developed in the serum agglutinins of the OXK type, were reinoculated with the homologous virus ; and that in 17 no significant rise of agglutinins developed.

Seven rabbits that, after inoculation of the "Manickam" strain of urban typhus, had developed in the serum agglutinins of the OX19 type, were reinoculated with the homologous virus ; no significant rise of agglutinins occurred.

Four rabbits that had reacted typically to inoculation with three tsutsugamushi strains by the development in the serum of OXK agglutinins were reinoculated with virus of the urban typhus strain. Agglutinins of the OX19 type, characteristic of infection with urban typhus, developed in all 4. The detailed results were as follows :

	Rabbits.			
	No. 1.	No. 2.	No. 3.	No. 4.
Maximum titre of OXK agglutinins	(45) 1/150	(28) 1/110	(42) 1/1925	(12) 1/220
Maximum titre of OX19 agglutinins	(18) 1/340	(17) 1/75	(14) 1/38	(20) 1/770

(Figures in brackets = number of days after inoculation.)

Conversely, 3 rabbits that had reacted typically to inoculation with virus of the urban typhus strain by the development of OX19 agglutinins in the serum were reinoculated with the virus of one or other of two tsutsugamushi strains. Agglutinins of the OXK type, characteristic of tsutsugamushi, developed in all 3. The detailed results were as follows :

	Rabbits.		
	No. 5.	No. 6.	No. 7.
Maximum titre of OX19 agglutinins	(14) 1/192	(18) 1/300	(25) 1/440
Maximum titre of OXK agglutinins	(38) 1/250	(18) 1/96	(19) 1/110

For the correct appreciation of these agglutination titres the following factors are important: Firstly, in the OXK strains a titre of 1/50–1/75 is inconclusive unless the curve of successive titres shows waxing and waning features, and a titre above 1/75 is positive; in the case of the OX19 strains the corresponding titres are lower, being 1/25 and 1/50 respectively (*vide* Part V). Secondly, the height of titre attained is not necessarily a measure of the virulence of the virus in the inoculum. As Felix (1933) has reported, the degree of agglutination attained depends in part on the susceptibility of individual rabbits. For example, 0·1 c.c. of heart-blood virus of the urban typhus strain was inoculated by us on one occasion into each of two rabbits by the intra-ocular route; the serum of both developed agglutinins of the OX19 type to maximum titres of 1/500 and 1/34 respectively, both maxima being reached on the 21st day after inoculation. On another occasion two rabbits were similarly inoculated with spotted fever virus; the sera developed agglutinins of the OX19 type to maximum titres of 1/340 and 1/50 respectively, both attained on the 13th day. In both pairs of rabbits the curve of successive titres showed waxing and waning features, and reinoculation with the homologous virus would have shown all to be completely immune.

In the cross-immunity results set out above, rabbits Nos. 1, 2, 4, 5, 6 and 7 showed a restimulation of agglutinins that were different in type from those previously stimulated, and that accorded with the antigenic type of the virus reinoculated. In rabbit No. 3 the OX19 agglutinins reached a maximum titre of only 1/38, but the waxing and waning features were present, and the agglutinogenic response was probably no less significant than that of the other six rabbits.

The above results show that between strains of tsutsugamushi and urban typhus in the rabbit there is no cross-immunity, whether the criterion of reinfection be clinical or serological.

The Monkey.

It was reported in Part VII that a gibbon and a macacus monkey that had recovered from infection with tsutsugamushi virus did not react when reinoculated with the same virus, save for the occurrence of a mild leucopenia in one of them. Later, both these animals were inoculated by the intradermal route with the virus of urban typhus. Infection resulted in both, the features of which were a mild febrile reaction, leucopenia, and the development in the serum of agglutinins for the OX19 strain of *Proteus* to significant titres; no skin-lesion occurred at the site of inoculation. These signs of infection corresponded precisely in number and degree with those observed following inoculation of a normal macacus monkey with the same urban typhus strain by the same route.

Summary of Cross-Immunity Experiments.

The foregoing results in the guinea-pig, rabbit and monkey are in entire accord in demonstrating the complete lack of cross-immunity between the strains of tsutsugamushi and urban typhus in these animals.

CONCLUSION.

Comparative study of strains of tsutsugamushi and urban typhus in laboratory animals has shown that in ætiology, experimental transmission, serology and experimental infections they differ entirely. Considered in conjunction with the contrasting epidemiology of the diseases, this study indicates conclusively that the two diseases are separate entities, as Fletcher and Lesslar (1926) first suggested on the grounds of serology and epidemiology.

PART IX: THE RELATION OF THE TSUTSUGAMUSHI DISEASE (INCLUDING RURAL TYPHUS) AND URBAN TYPHUS TO ROCKY MOUNTAIN SPOTTED FEVER.

Parker and Davis (1933*a*) and Dyer (1933) in the United States of America, and Monteiro (1933) in Brazil, have demonstrated the immunological identity of Rocky Mountain spotted fever* and the typhus of Sao Paulo. Parker and Davis (1933*b*), employing protection tests, obtained results that suggest the lack of any close relationship between tsutsugamushi and South African tick-bite fever on the one hand, and spotted fever on the other hand. The relationship of the *fièvre boutonneuse* and spotted fever was investigated by Badger (1933) in the United States of America, and by Brumpt (1932) in France. The former regards the two diseases as closely allied, the latter worker as differing. The clear distinctions that exist between spotted fever and the endemic typhus of the United States of America were demonstrated by Dyer and his associates in a succession of papers.

Nicolle and Laigret (1933) concluded as a result of cross-immunity experiments that the virus of spotted fever differed from the viruses of " typhus historique " and " typhus murin ".

The collaboration of this Institute was invited by Dr. R. R. Parker (of the Rocky Mountain Spotted Fever Laboratory, Hamilton, Montana, U.S.A.), with a view to an investigation of the relationship of the typhus-like fevers of Malaya with spotted fever. The method of exchange of the respective viruses was as follows. Nymphs and adults of the wood tick, *Dermacentor andersoni*, infected with the virus of spotted fever, were received from Hamilton and successfully " reactivated ". Since tissue-virus of the two types of Malayan typhus will not survive transportation of 11 days' duration, transportation in an insect vector was attempted ; and for this purpose Dr. Parker kindly forwarded to this laboratory a supply of uninfected *Dermacentor andersoni* ticks. These were allowed to feed on infected guinea-pigs of the " Seerangayee" strain of tsutsugamushi and the " Manickam " strain of urban typhus, were then removed, and one-half their number returned to Hamilton for re-activation there, the other half being retained for investigation by us. Nymphs and adults of *Rhipicephalus sanguineus*, the commonest Malayan tick, were similarly fed and despatched.

In Part VI of this series (1936*c*) the results of attempted experimental transmission of the tsutsugamushi disease and urban typhus by the agency of the ticks retained by us were described in detail. The present paper records

* Referred to in the text, for brevity, as " spotted fever ".

the establishment of a strain of spotted fever in the guinea-pig, and the results of cross-immunity tests between this strain and the Malayan typhus-like viruses, of certain " protection " tests, and of attempted vaccination of guinea-pigs by spotted-fever vaccine.

EXPERIMENTAL SPOTTED FEVER IN THE GUINEA-PIG.

Ten infected adult ticks (*D. andersoni*) that had been fed at Hamilton for 2 to 3 days prior to despatch were placed for re-activation on to an uninfected guinea-pig (for technique, *vide* Part VI). Six of the ticks attached, and the guinea-pig reacted, a febrile reaction beginning on the fifth day. By passage of heart-blood virus the disease was reproduced in a second generation of guinea-pigs ; and no difficulty was experienced in maintaining the strain by similar passage. The strain was maintained for 45 generations, and then discarded. Fever occurred usually on the third to fifth day after inoculation ; scrotal swelling developed in 90 p.c. of males ; the mortality was 50 p.c. when whole blood was the inoculum, and 40 p.c. when serum was substituted.

CROSS-IMMUNITY EXPERIMENTS IN GUINEA-PIGS.

Twelve guinea-pigs that had recovered from infection with spotted-fever virus were reinoculated with the homologous virus ; all proved to be immune.

Tsutsugamushi and Spotted Fever.

Fifteen guinea-pigs that had recovered from infection with the virus of the " Seerangayee " guinea-pig strain of tsutsugamushi were reinoculated with the spotted fever virus. All reacted with fever ; 9 of the 11 that were males reacted also with scrotal swelling. Of 7 inoculated with whole-blood virus, 5 died ; of 8 inoculated with serum virus, 3 died ; so that the mortality was approximately that of passage guinea-pigs. Conversely, 13 guinea-pigs that had recovered from infection with the virus of spotted fever were reinoculated with the virus of the " Seerangayee " strain. One only failed to react ; 12 reacted with characteristic fever ; 7 of the 12 died of the infection, so that the mortality was that of passage guinea-pigs.

There is thus absence of cross-immunity between the viruses of spotted fever and tsutsugamushi as demonstrated by experiments in guinea-pigs.

Urban Typhus and Spotted Fever.

Twenty guinea-pigs that had recovered from infection with the " Manickam " strain of urban typhus were reinoculated with the virus of spotted fever. Of these, 3 failed to react ; 17 reacted with fever ; 8 of the 14 that were males developed typical scrotal swelling. Of 13 inoculated with whole-blood virus, only 2 died ; of 7 inoculated with serum virus, none died ; so that the mortality due to the spotted-fever virus was much reduced in comparison with that of passage guinea-pigs. Conversely, 20 guinea-pigs that had recovered from infection with the virus of spotted fever were reinoculated with the virus of the urban typhus strain. Of these, 6 entirely failed to react ;

14 showed a febrile reaction that was mild and brief; 2 only showed a fever that in intensity and duration could be regarded as characteristic of the urban typhus strain. In none of the 18 that were males did scrotal swelling occur—a finding that is in marked contrast with the occurrence of scrotal swelling in fully 70 p.c. of male passage guinea-pigs of the " Manickam " strain. As also in passage animals of the " Manickam " strain, there was no mortality.

Thus, as demonstrated in guinea-pigs, there was absence of cross-immunity between the viruses of spotted fever and urban typhus in the majority (17 out of 20) of instances when the second inoculum was the virus of spotted fever; but apparently a very definite though incomplete cross-immunity when the second inoculum was the virus of urban typhus.

A similar finding concerning the reduced mortality noted above was that of Nicolle and Laigret, who inoculated spotted fever virus into guinea-pigs immune to " typhus historique " and to Mexican typhus, and found no immunity, but a diminution in death-rate.

CROSS-IMMUNITY EXPERIMENTS IN RABBITS.

Cross-immunity experiments complementary to those in guinea-pigs were carried out in rabbits. It was found that the reaction to intra-ocular inoculation of spotted-fever virus closely resembled that caused by the inoculation of the virus of urban typhus, viz. a mild and fleeting iridocyclitis, occurring in about one-half the cases (16 out of 26), that could not be maintained beyond three generations. The majority of rabbits (15 out of 19), however, responded with a positive Weil-Felix reaction, agglutination being always of the OX19 strain of *Proteus*, never of the OXK strain. (Tests were not carried out with the OX2 strain of *Proteus*.) The inconstancy of the ocular reaction detracts from its use as a criterion of successful infection with spotted-fever virus. The development of a positive Weil-Felix reaction is a more constant, and therefore more reliable, criterion; and was relied on in the experiments reported below. The comments relating to the standards of the positive agglutination of the OX19 type that is caused by urban typhus virus apply equally, in our experience, to the OX19 agglutination caused by spotted-fever virus.

Tsutsugamushi and Spotted Fever.

Five rabbits that had reacted to intra-ocular inoculation of the virus of tsutsugamushi, by evolution of ocular signs and by development of agglutinins to the OXK strain of *Proteus*, were reinoculated into the unused eye with serum of reacting guinea-pigs of the spotted-fever strain. The serological findings were as follows:

	Rabbits.				
	No. 8.	No. 9.	No. 10.	No. 11.	No. 12.
Maximum titres of OXK agglutinins	(15) . 1/500	(38) . 1/58	(55) . 1/220	(42) . 1/1925	(21) . 1/250
Maximum titres of OX19 agglutinins	(10) . 1/28	(7) . 1/19	(12) . 1/1925	(13) . 1/340	(13) . 1/50

(Figures in brackets = number of days after inoculation.)

No rise of agglutinins for the OXK strain resulted ; but agglutination of the OX19 strain developed to significant titres in rabbits Nos. 10, 11 and 12, but was inconclusive in rabbits Nos. 8 and 9.

Conversely, 6 rabbits that had reacted to intra-ocular inoculation of the virus of spotted fever by development of significant agglutination of the OX19 strain were reinoculated intra-ocularly with the virus of tsutsugamushi. All 6 developed the full ocular reaction typical of tsutsugamushi strains. One rabbit died before agglutination of the OXK type could be expected to develop. The serological findings were as follows :

	Rabbits.				
	No. 13.	No. 14.	No. 15.	No. 16.	No. 17.
Maximum titres of OX19 agglutinins	(17) 1/170	(16) 1/282	(15) 1/282	(16) 1/34	(13) 1/19
Maximum titres of OXK agglutinins	0	0	(38) 1/250	(22) 1/85	0

No rise of agglutinins for the OX19 strain resulted ; in rabbits Nos. 15 and 16 the serum developed significant agglutination of the OXK strain ; in the others no development of OXK agglutinins occurred. (In connection with the Weil-Felix reactions, it is recalled that in approximately 60 p.c. of the passage rabbits of the tsutsugamushi strains the serum during convalescence agglutinates the OXK strain ; and that, in contrast, the OX19 strain is not agglutinated.)

The above findings confirm the complementary results in guinea-pigs, viz. that between the viruses of spotted fever and tsutsugamushi there is absence of cross-immunity, and thus accord with the known differences in epidemiology, ætiology, serology and experimental infections.

Urban Typhus and Spotted Fever.

Three rabbits that had reacted to intra-ocular inoculation of the virus of the " Manickam " strain of urban typhus by development of significant agglutination of the OX19 strain, were reinoculated after the fall of agglutination to zero with serum virus of spotted fever by the intra-ocular route. The detailed results were as follows :

		Rabbits.		
		No. 18.	No. 19.	No. 20.
Infection with urban typhus	Maximum titre of OX19 agglutinins	(18) 1/250	(23) 1/192	(18) 1/38
Infection with spotted fever	Maximum titre of OX19 agglutinins	(17) 1/440	(13) 1/22	(17) 1/125

Thus, in all 3, agglutination of the OX19 strain recurred, in Nos. 18 and 20 to a higher titre than the original OX19 titre. Rabbit No. 19 died on the 15th day after inoculation, *i. e.* before the maximum titre could be known.

Conversely, 5 rabbits that reacted to intra-ocular inoculation of the virus of spotted fever with significant agglutination of the OX19 strain were reinoculated, after the fall of the titre to zero, with the virus of the " Manickam "

strain of urban typhus by the intra-ocular route. The detailed results were as follows :

		Rabbits.				
		No. 21.	No. 22.	No. 23.	No. 24.	No. 25.
Infection with spotted fever	Maximum titre of OX19 agglutinins	(13) 1/340	(13) 1/50	(16) 1/34	(15) 1/282	(14) 1/96
Infection with urban typhus	Maximum titre of OX19 agglutinins	(14) 1/38	0	0	(14) 1/192	0

Thus development of OX19 agglutinins recurred in 2 only, to a significent titre in No. 24, and to an inconclusive though probably significant titre in No. 21.

These results in rabbits were consistent with those noted above in experiments with guinea-pigs, viz. that when the animal was convalescent from urban typhus and was reinoculated with the spotted fever strain, no cross-immunity could be demonstrated (apart from a reduced mortality in guinea-pigs) ; and that in contrast, the converse experiments suggested that a partial cross-immunity existed. This minor degree of cross-immunity apart, urban typhus and spotted fever are separate entities, distinct in their epidemiology, ætiology, serology and experimental infections.

THE PROTECTIVE VALUE OF SPOTTED FEVER VACCINE.

Spencer and Parker (1930) have prepared a highly efficacious vaccine against spotted fever, that consists of tick-tissue virus chemically killed by phenol and formalin. Guinea-pigs that have received a single subcutaneous inoculation of this vaccine prove to be immune to a test inoculation with spotted-fever virus given 10 days later. Moreover, in the course of an investigation into the relationship of the Sao Paulo typhus virus and spotted-fever viruses, Davis and Parker (1933) have demonstrated that this vaccine is equally efficacious in rendering guinea-pigs immune to experimental infection with Sao Paulo typhus virus.

At Dr. Parker's suggestion, similar tests were undertaken at this Institute with the Malayan strains. Ten guinea-pigs were vaccinated ; all proved to be immune to a test inoculation of spotted-fever virus made 10 days later. Eight guinea-pigs were vaccinated, and inoculated 10 days later with virus of the " Seerangayee " strain of tsutsugamushi ; all 8 reacted ; 4 died of the infection. Eight guinea-pigs were vaccinated, and inoculated 10 days later with the virus of the " Manickam " strain of urban typhus ; all 8 reacted ; 4 out of 6 that were males developed also a typical scrotal reaction. Thus the vaccine had no protective power against the viruses of tsutsugamushi and urban typhus.

PROTECTION TESTS WITH CONVALESCENT SERUM.

Parker and his co-workers have shown that serum drawn from man or laboratory animal during convalescence following an infection with spotted fever has a protective value against spotted-fever serum-virus. They consider

protection tests to be of diagnostic value, and undertake them as an aid to diagnosis where indicated. They have made corresponding tests with convalescent serum from guinea-pig and man recovered from either tsutsugamushi or urban typhus. In a personal communication Dr. Parker states that no protection against spotted-fever serum-virus was given by either type of convalescent serum.

Complementary to this investigation, tests were carried out by us in this laboratory to determine the protective value of convalescent guinea-pig sera of the two forms of Malayan typhus against the virus of the respective homologous strain. On various occasions convalescent guinea-pig or human serum from cases of tsutsugamushi was added to blood-virus and allowed to stand at room temperature for half an hour ; the mixture was then inoculated intraperitoneally into a group of guinea-pigs. In all, a febrile reaction followed that differed in no significant detail from that observed in control animals. Similar tests were carried out with convalescent serum of guinea-pigs recovered from infection with urban typhus. Occasionally a delayed incubation period or aborted febrile reaction was observed ; but in many cases the reaction occurred precisely as in controls. Such degree of protection that was afforded was so mild and inconstant as to preclude such a test from having diagnostic value.

Since convalescent serum from tsutsugamushi or urban typhus cases has little or no protective value against the homologous virus, the protection test cannot be used as a differential criterion between strains of spotted fever and strains of the two Malayan typhus-like fevers.

SUMMARY.

Part VIII.

1. Cross-immunity experiments in the guinea-pig, rabbit and monkey were carried out with the viruses of the tsutsugamushi disease (including rural typhus) and the urban typhus of Malaya ; they showed that immunogenically the two viruses are distinct.

2. The characteristics of ætiology, epidemiology, serology and experimental infections are compared, and the conclusion drawn that the two diseases belong to entirely separate groups of rickettsial disease.

Part IX.

1. Cross-immunity experiments in the guinea-pig and rabbit were carried out with the viruses of Rocky Mountain spotted fever, tsutsugamushi (including rural typhus) and urban typhus. They showed that, immunologically, tsutsugamushi and spotted fever are entirely distinct ; whereas urban typhus and spotted fever, though more distinct than alike immunologically, do possess a minor degree of reciprocal cross-immunity.

2. Spotted-fever vaccine was found to have no protective value against the viruses of tsutsugamushi and urban typhus.

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REFERENCES.

ANIGSTEIN, L.—(1933) "Studies on Tropical Typhus," *Stud. Inst. med. Res. F.M.S.*
BADGER, L. F.—(1933) *Publ. Hlth. Rep. Wash.*, **48**, 507.
BRUMPT, E.—(1932) *C. R. Soc. Biol.*, Paris, **110**, 1197.
DAVIS, G. E., AND PARKER, R. R.—(1933) *Publ. Hlth. Rep. Wash.*, **48**, 1006.
DYER, R. E.—(1933) *Ibid.*, **48**, 521.
FELIX, A.—(1933) *Trans. R. Soc. trop. Med. Hyg.*, **26**, 365.
FLETCHER, W., AND FIELD, J. W.—(1927) *Bull. Inst. med. Res. F.M.S.*, No. 1 of 1927.
Idem AND LESSLAR, J. E.—(1925) *Ibid.*, No. 2 of 1925.—(1926) *Ibid.*, No. 1 of 1926.
FLETCHER, W., LESSLAR, J. E., AND LEWTHWAITE, R.—(1929) *Trans. R. Soc. trop. Med. Hyg.*, **23**, 57.
LEWTHWAITE, R., AND SAVOOR, S. R.—(1936*a*) *Brit. J. exp. Path.*, **17**, 1.—(1936*b*) *Ibid.*, **17**, 23.—(1936*c*) *Ibid.*, **17**, 208.—(1936*d*) *Ibid.*, **17**, 214.
Idem, HODGKIN, E. P., AND SAVOOR, S. R.—(1936*e*) *Ibid.*, **17**, 309.
MONTEIRO, J. L.—(1933) *Brazil-med.*, **25**, 437.
NICOLLE, C., AND LAIGRET, J.—(1933) *Arch. Inst. Pasteur, Tunis*, **22**, 36.
PARKER, R. R., AND DAVIS, G. E.—(1933*a*) *Publ. Hlth. Rep. Wash.*, **48**, 1006.—(1933*b*) *Ibid.*, **48**, 839.
SPENCER, R. R., AND PARKER, R. R.—(1930) *U.S. Publ. Hlth. Serv., Hyg. Lab. Bull.*, **154**.

THE NECESSITY OF LIVING CELLS FOR THE CULTIVATION OF PSITTACOSIS VIRUS.

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THE question of whether or not living cells are essential to the multiplication of viruses is of considerable importance. If one excludes the agents of bovine pleuro-pneumonia and agalactia of sheep and goats from the virus group—and there seems to be good reason for doing this, at any rate for the time being—the bulk of evidence suggests that the viruses are highly specialized parasites requiring for their reproduction the environment found only within living cells. From time to time, however, the claim to have succeeded in

growing a virus in a cell-free medium has been advanced. In this respect the work of Eagles and his colleagues with vaccinia virus is the most recent and most impressive (Eagles and McClean, 1930–1931 ; Eagles and Kordi, 1932 ; Eagles, 1935), but this work has not been confirmed (Maitland, Lang and Lyth, 1932 ; Rivers and Ward, 1933*a* and *b*).

For the further investigation of the question there is no doubt that psittacosis virus possesses qualities which make it peculiarly suitable. It can be stained comparatively easily, and being readily seen under the microscope, it should be possible to combine histological study with virus titrations in the examination of cultures of the virus made with living cells. This should enable one to find out if the virus does occasionally grow outside cells, and if so, then one would approach the problem of cultivation apart from living cells with greater confidence and more hope of success.

EXPERIMENTAL.

Cultivation with Living Cells. Fractional Examination of the Cultures.

Infection of the tissue.—The first experiment was designed to show if in a tissue culture infected with psittacosis virus there was any evidence of extra-cellular multiplication of the virus. The cultures were put up in small cells made by luting sterile glass rings (2 cm. diam. × 1 cm. deep) to sterile glass slides with melted paraffin. Muscle from 13-day chick embryos was cut into fragments of a suitable size and soaked in suspensions of psittacosis virus (virulent mouse spleen suspension centrifuged at 10,000 r.p.m. for 1 hour, and deposit resuspended in Pannet-Compton saline) for 1 hour at 37° C. The tissue fragments were then washed four times in Pannet-Compton saline and distributed in the glass culture-cells, 5 to 6 pieces per cell.

They were then covered with 2 drops of hen plasma and 4 drops of chick embryo extract (one 10-day chick embryo to 5·0 c.c. Pannet-Compton saline), and when the clot had formed, 1·0 c.c. embryo extract was run in on top. The cells were sealed with a cover-slip by means of the usual paraffin-vaseline mixture. Thirty-two cultures were put up at a time. Eight of these were examined at once and the remainder were placed in the incubator at 37° C. At intervals of 24, 36 and 48 hours cultures were withdrawn from the incubator and examined for the presence of virus. In each examination, whether immediate or after periods of incubation, the procedure was the same. The fluid portion of the culture was pipetted off and pooled. The clots, together with the tissue they contained, were then scraped off and ground up in a mortar with sand and taken up in 1·0 c.c. saline. The clots from 4 cultures in each batch were used for the purpose of histological examination. After fixation with methyl alcohol they were stained overnight with Giemsa (1 drop Giemsa per 1 c.c. Giemsa buffer ; differentiation with glycerine-ether, 1 : 5) and examined microscopically in order to see how much virus was present, the form it was in, and how it was distributed, whether intra- or extra-cellular. Mice were inoculated with the fluid portion of the cultures and with the suspension of the clots, 2 mice being used for each lot of material, the dose inoculated being 0·5 c.c. intraperitoneally.

These mice were kept under observation for a period of 3 to 4 weeks, and then any survivors were killed and examined for evidence of psittacosis infection.

TABLE I.

Examination of cultures for presence of virus.

Experiment.	Portion of culture.	By mouse inoculation, immediately or after—				By the microscope (clot only) after—		
		Immediately.	24 hours.	36 hours.	48 hours.	24 hours.	3 hours.	48 hours.
1	Fluid	D 8 +	D 21 −	..	D 23 −	Few large forms. No free virus	..	Mostly small forms. Moderate amount. No free virus.
		D 10 +	K 24 −	..	D 23 −			
	Clot	D 12 +	D 10 +	..	D 5 +			
		K 19 +	D 16 +	..	D 6 +			
2	Fluid	D 7 +	D 12 +	..	D 5 +	Large forms moderate amount. No free virus	..	Intermediate and small forms. Large amount. No free virus.
		D 8 +	D 13 +	..	D 6 +			
	Clot	D 7 +	D 6 +	..	D 5 +			
		D 12 +	D 6 +	..	D 6 +			
3	Fluid	K 22 −	K 24 −	..	D 5 +	Ditto	..	Ditto.
		K 22 −	K 28 −	..	D 7 +			
	Clot	K 22 −	D 5 +	..	D 3 +			
		K 22 −	D 5 +	..	D 3 +			
4	Fluid	D 9 +	D 11 +	..	D 7 +	„	..	Mainly small forms. Very large amount. Some cells packed.
		D 21 +	D 12 +	..	D 8 +			
	Clot	D 12 +	D 4 +	..	D 8 +			
		D 21 +	D 5 +	..	D 4 +			
5	Fluid	D 8 +	K 26 −	..	..	Large forms numerous. No free virus		
		K 27 −	K 26 −					
	Clot	D 8 +	D 6 +					
		D 9 +	D 9 +					
6	Fluid	D 6 +	D 12 −	..	..	Large forms moderate amount. No free virus		
		K 27 −	D 19 −					
	Clot	D 5 +	D 9 +					
		D 5 +	D 9 +					
7	Fluid	D 6 +	D 9 +	..	..	Large forms numerous. No free virus		
		D 7 +	K 28 −					
	Clot	D 6 +	D 4 +					
		D 7 +	D 6 +					
8	Fluid	K 28 −	K 27 −	..	..	Large forms moderate amount. No free virus		
		K 28 −	K 27 −					
	Clot	K 28 −	D 7 +					
		K 28 −	D 10 +					
9	Fluid	D 8 +	D 11 +	K 21 −	..	Large forms few. No free virus	Large and intermediate forms. No free virus	
		D 10 +	K 22 −	K 21 −				
	Clot	D 5 +	D 9 +	D 4 +				
		K 22 −	D 10 +	D 4 +				
10	Fluid	D 8 +	K 20 −	D 6 +	..	Large forms moderate amount. No free virus.	Large, intermediate and small forms. No free virus	
		K 20 −	K 20 −	D 6 +				
	Clot	D 8 +	D 5 +	D 3 +				
		K 20 −	K 20 −	D 4 +	..			

D = died. K = killed for examination though in good health. The number indicates the period in days between inoculation and death or examination of the animal. + = virus demonstrated microscopically. − = no virus found.

For this purpose the microscopic examination of smears made from likely material—spleen principally—and stained by Castañeda's method was relied on. All mice dying earlier were, of course, examined in a similar manner. The findings in 10 such experiments are recorded in Table I.

One rather unexpected result is the amount of virus present in the fluid portions of the cultures immediately after they have been put up. One had anticipated finding only traces of virus there, insufficient possibly to bring about the death of the inoculated mice, and had this been so, then after subsequently estimating the amount of virus in the fluid and solid portions of the cultures after varying periods of incubation and correlating this evidence with the histological evidence, it should have been possible to get a clear indication as to whether virus multiplied only in the cells. Unfortunately this anticipation was not realized. Even when the tissue fragments which had been soaked in virus were washed as many as 20 times, virus was still coming away in the washing fluid, so that the desideratum—a virus-free fluid part of the culture—was not realized.

However, the results from even this imperfect type of experiment do suggest that the virus grows only inside the cells or in intimate association with them, since without exception the virus, present in the supernatant fluid at the outset, has diminished or disappeared after 24 hours' incubation, reappearing later, whereas in the solid portion of the culture, virus increased steadily in quantity from the beginning. In Experiments 3 and 8 this is particularly clearly shown; in fact the former experiment almost attains the ideal at which we aimed. In no instance did microscopical examination of the clot reveal virus outside cells.

Infection of the supernatant.—It was thought that possibly if the virus was introduced into the fluid portion of the culture rather than adsorbed on the tissue, a clearer result might be obtained. Presumably, if this were done a small portion of the virus would find its way to the cells, setting up a minimal infection there. Meanwhile the bulk of the virus in the supernatant fluid would die and be replaced later when multiplication in the cells had advanced far enough to supply free virus. Accordingly cultures were put up as in the first experiment with the exception that the tissue fragments were not infected. When the plasma-clot had formed around the tissue, embryo extract, infected by suspending in it virus centrifuged out of a filtrate of virulent mouse spleen, was run in on top. The cultures were incubated at 37° C. The fluid portion of batches of these cultures was tested for the presence of virus by mouse inoculation after varying periods of incubation and the clots were examined microscopically after fixation and staining with Giemsa; a portion of the infected embryo extract served to control the initial virus concentration. The results in these experiments are recorded in Table II.

Here, again, the evidence is inconclusive. In the first three experiments there is a suggestion that the virus content of the fluid part of the cultures has undergone some diminution during the first 48 hours of incubation, and in Experiment 3, which was prolonged for 72 hours, there is an indication that the virus content of the supernatant embryo extract is beginning to increase again by the 3rd day. The persistence of virus in the fluid part of the cultures in these experiments would seem to depend on the presence of living cells in the

D = died. K = killed for examination though in good health. The number indicates the period in days between inoculation and death or examination of the animal. + = virus demonstrated microscopically. − = no virus found.

TABLE II.

Experiment	Test for presence of active virus in fluid part of culture immediately or after—				Histological examination of clot after—		
	Immediately.	24 hours.	48 hours.	72 hours.	24 hours.	48 hours.	72 hours.
1	D 3 +	D 6 +	..	..	Tissue grew well. No virus seen	..	..
	D 3 +	D 7 +					
2	D 2 +	D 6 +	D 6 +	..	Large forms in fair number	Large and intermediate forms moderate amount.	
	D 3 +	D 6 +	D 6 +				
3	D 3 +	D 7 +	D 6 +	D 5 +	No virus seen	Small amount of virus seen. All in cells	Large amount of virus seen. All in cells.
	D 4 +	D 7 +	D 6 +	D 5 +			
4	D 3 +	D 7 +	S	D 12 +	„	Small amount of virus. All in cells	Moderate amounts of virus. All in cells.
	D 5 +	S	S	D 16 +			
5	D 5 +	S	S				
No tissue in plasma clot	D 5 +	S	S				
6	D 5 +	D 10 +	S				
Boiled tissue in plasma clot	D 5 +	D 11 +	S				
7	D 4 +	D 4 +	D 8 −*				
Boiled tissue in plasma clot	D 7 +	D 6 +	S				

* Died from intercurrent disease. S = survived.

clot, since in the control experiment No. 5, where the clot contained no tissue, the virus had disappeared in 24 hours. And yet it would not seem to be a question of virus multiplication in conjunction with the tissue, since in only one of the three experiments was any virus demonstrable microscopically in the clots after 24 hours' incubation. Further, the control experiments Nos. 6 and 7 showed that the presence of the boiled tissue in the plasma-clot enabled the virus to persist for longer than 24 hours in the supernatant fluid. Boiled tissue would influence the reduction potential in the fluid overlying the clot, though to a less extent than the living cells, and it would seem probable that the persistence of the virus in Experiments 1, 2 and 3 is due to prolonged survival rendered possible by the maintenance of the reduction potential at an optimum level. These three experiments, therefore, give us little information of value. With a smaller initial inoculum, however, as in Experiment 4, virus had disappeared from the fluid part of the cultures by 48 hours. Histological examination of the clots after this period of incubation showed that infection of the cells had taken place; a small amount of intracellular virus could be seen. Later, after 72 hours' incubation, the virus in the tissue had increased considerably and virus was again present in the fluid part of the cultures. The evidence supplied by this last experiment definitely favours the view that the virus multiplies in the cells.

Cultures in which the Infected Embryo Extract was Separated from the Plasma Clot and Tissue by a Permeable Collodion Membrane.

Although the results given by the above two types of experiment support the view that psittacosis virus is an obligate intracellular parasite, they have not excluded the possibility that it may occasionally be capable of growing extracellularly. In an attempt to throw further light on this point a third type of experiment was made. A very similar method has been employed by Muckenfuss and Rivers (1930) and by Muckenfuss (1931) in the investigation of the cultural requirements of vaccinia virus. The growing chick embryo cells were separated from the fluid part of the culture containing the virus by a collodion membrane permeable to protein, but impermeable to virus. Under these conditions proteins and substances of smaller molecular size should be able to pass through to the embryo extract containing the virus, but the virus would not have access to the cells. The membranes were made of a 2 p.c. nitrocellulose in a solvent consisting of 75 parts of absolute alcohol and 25 parts of ether. Collodion was poured on to a clean glass surface, any excess being allowed to drain away, and after evaporation for 15 minutes the membranes were taken off and washed well in water. Pieces of membrane cut to the requisite size were luted by means of collodion to glass rings of a similar size to those used in making the culture cells. These were then well washed and sterilized by steaming for 30 minutes. Culture cells similar to those used in the first two types of experiment were prepared, and in these chick embryo tissue was implanted in plasma as before. The cell was then filled up with chick embryo extract. Placed on top of this was the second glass ring carrying the collodion membrane, with the membrane in contact with the culture cell. The two rings were luted together by means of sterile vaseline. In this way a double cell was formed, separated by the intervening collodion membrane.

TABLE III.

Experiment.	Virus used.	Test for virus in upper cell, immediately or after—					Histological examination of clot after—			
		Immediately.	24 hours.	30 hours.	45 hours.	68 hours.	24 hours.	30 hours.	45 hours.	68 hours
1	Virus concentrated from virulent mouse spleen suspension by centrifugation	D 2	..	..	..	D 9	..	..	..	No virus seen.
		D 2	..	..	..	D 9				
2	As in Experiment 1	D 3	S	..	..	..	No virus seen			
		D 3	S							
3	Gradocol filtrate of suspension of virulent mouse spleen	D 7	S	..	..	..	„			
		D 7	S							
4	As in Experiment 3	D 5	S	S	..	..	„	No virus seen		
		D 5	S	S						
5	Supernatant from a suspension of virulent mouse spleen	D 4	D 5	D 6	D 7	..	„	„	No virus seen	
		D 4	D 5	D 6	D 7					
6	As in Experiment 5	D 1	D 5	S	..	..				
		D 1	D 5	S						

The lower of the two cells contained the tissue culture and was filled up to the membrane with embryo extract. In the upper cell, above the membrane, virus suspended in embryo extract was placed, and the cell was sealed with a coverslip and paraffin-vaseline mixture. Representative samples of the membranes had been tested for permeability to proteins, but, in addition, each batch of cultures put up contained controls in which horse-serum replaced the virus in the upper chamber. At the end of the experiment the presence of horse-serum in the fluid of the lower chamber of these controls was tested for by means of an anti-horse precipitating serum ; in every case horse-serum had passed through the membrane to the lower chamber. Six experiments of this kind were carried out. After varying periods of incubation at 37° C. cultures were examined for the presence of virus in the upper cell by mouse inoculation, and the clot in the lower cell was fixed and stained and examined microscopically for tissue growth and the presence of virus. Mice inoculated with the virus suspension in embryo extract served to control the initial virus concentration. Two mice were inoculated intraperitoneally, 0·5 c.c. each, with each lot of material. The results of these experiments are given in Table III.

In only two of the experiments did the virus survive longer than 24 hours under these conditions of cultivation, and in both the results suggest that its persistence in the fluid in the upper cell was due to prolonged survival rather than to multiplication. In these two experiments, Nos. 1 and 5, the virus used had not been filtered and must have contained fragments of mouse spleen cells, though it is unlikely that any whole viable cells were present. It is possible, though unlikely, that the persistence of the virus in these two experiments was due to some multiplication rendered possible by the presence of tissue fragments, but not proceeding on a scale high enough to make good the loss from virus death. In view of this remote possibility, it was decided to carry out some experiments with a fresh tissue extract as culture medium on the lines which had given Eagles his successful results with vaccinia virus.

Attempts at Cultivation in a Cell-free Medium.

Composition of medium.—Two 13 to 14-day-old chick embryos were cut up finely with scissors in a large centrifuge tube, and 1·0 c.c. 9 p.c. salt solution was added and mixed thoroughly with the mince. After standing for 1 hour in the refrigerator (± 4° C.) 9·0 c.c. of sterile distilled water was added, mixed with the tissue mince, and the tube was then centrifuged at 2000 r.p.m. for 45 minutes. At the end of this time practically all the tissue had been deposited. Immediately overlying the tissue deposit the fluid was murky, and on microscopic examination was seen to contain a few whole cells. The upper part of the column of fluid—about 7·0 c.c.—was cloudy and contained cell *débris* and granules, but no intact cells ; it was this portion which was pipetted off and used as the tissue extract in the culture medium. The other ingredients of the medium were fowl serum and Pannet-Compton saline.

Virus.—A virulent mouse spleen was ground up and suspended in 5·0 c.c. Pannet-Compton saline. After this had been kept in the refrigerator for 1 hour the top 3·0 c.c. of fluid were pipetted off and used for sowing the cultures. Microscopical examination did not show any whole cells in the virus suspension so prepared.

Set-up of cultures.—The ingredients were mixed in bulk in a sterile flask in the following proportions: virus suspension 1 part, fowl's serum 1 part, tissue extract 6 parts, Pannet-Compton saline 2 parts. After thorough mixing the mixture was pipetted out into Carrel D flasks, 2·0 c.c. per flask. Cultures were incubated at 37° C., and a portion of the mixture was kept in the refrigerator for testing for initial virus concentration.

Titration of cultures.—This was carried out in the rabbit skin. Falling decimal dilutions were inoculated intradermally in 0·2 c.c. amounts. Although this is a less sensitive method of testing for the presence of virus than mouse inoculation, it enables the whole titration to be carried out in one animal, thus overcoming the disadvantage of varying susceptibility of individual animals. The reactions produced by this virus in the rabbit skin are very definite, deep red in colour and raised well above the surface; they begin to appear 48 hours after inoculation and reach their maximum by the 3rd day. An endeavour was made to start each experiment with a mixture which, when diluted 1 in 10 and injected intradermally in a rabbit's skin in a dose of 0·2 c.c., would give a definite reaction. Wherever this was not achieved or the result in the rabbit's skin was at all doubtful, mice were inoculated with the unincubated mixture to make sure that active virus was, in fact, present. The cultures were titrated after 3 days' incubation at 37° C., and the results of these experiments are recorded in Table IV.

TABLE IV.

Experiment.	Type of virus used.	Titre in rabbit's skin.		Infectivity of unincubated culture for the mouse.
		Unincubated.	After 3 days at 37° C.	
1	Elementary bodies	<1/10	<1/10	No illness. Mice tested later and found immune
2	,,	1/1000	<1/10	..
3	,,	1/1000	<1/10	..
4	,,	1/1000	<1/10	..
5	Large forms	<1/10	<1/10	No illness. Mice tested later and found immune.
6	,,	<1/10	<1/10	Ditto
7	,,	1/10	<1/10	D 7 D 7
8	,,	1/10	<1/10	D 12 D 12

In the first four experiments the virus implanted in the cultures was almost exclusively in the form of elementary bodies. In three out of the four experiments the initial concentration of the virus was high, giving a reaction in the rabbit's skin when diluted 1 in 1000, but in the fourth (No. 1) it fell below 1 in 10, and only immunized mice without producing obvious illness. In every case, however, after 3 days' incubation the virus concentration was below 1 in 10. The failure in these experiments prompted the attempt to start the cultures with virus in the large forms. On the assumption that the large forms represented the vegetative stage of the virus, it was thought that possibly in this form the virus might multiply under conditions which the elementary bodies

found unsuitable. The next four experiments were therefore made with the virus in this form, but, as will be seen from the table, no greater success attended this venture. In this connection it is interesting to record that attempts to infect tissue cultures with virus in the large form have invariably failed, presumably because the large forms find it difficult to enter cells. This is in keeping with the observation of Bedson (1933) that the large form is less virulent for the mouse than the elementary body.

SUMMARY.

The evidence present in this paper supports the view that the virus of psittacosis is an obligate intracellular parasite.

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REFERENCES.

BEDSON, S. P.—(1933) *Brit. J. exp. Path.*, **14**, 267.
EAGLES, G. HARDY.—(1935) *Ibid.*, **16**, 188.
Idem AND KORDI, A. H. H.—(1932) *Proc. Roy. Soc.*, B, **111**, 329.
Idem AND MCCLEAN, D.—(1930) *Brit. J. exp. Path.*, **11**, 337.—(1931) *Ibid.*, **12**, 97.
MAITLAND, H. B., LANG, A. W. AND LYTH, R. —(1932) *Ibid.*, **13**, 90.
MUCKENFUSS R. S.—(1931) *J. exp. Med.*, **53**. 377.
Idem AND RIVERS, T. M.—(1930) *Ibid.*, **51**, 149.
RIVERS, T. M., AND WARD, S. M.—(1933*a*) *Ibid.*, **57**, 51.—(1933*b*) *Ibid.*, **57**, 741.

A NUTRIENT MIXTURE SUITABLE FOR THE GROWTH OF *STAPHYLOCOCCUS AUREUS.*

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IT has been shown previously (Knight, 1935) that *Staphylococcus aureus* can be grown aërobically in a mixture consisting of glucose, salts, hydrolysed gelatine reinforced with cystine, tyrosine and tryptophan and a "staphylococcus factor" the nature of which was partially elucidated. We have studied the nutritional requirements of this organism further and find that growth can be obtained aërobically without any adaptation or "training" in a medium whose chemical composition is known except in respect of its content of

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"staphylococcus factor" (Knight, 1935). For anaërobic growth an additional unknown is necessary under the conditions used which is at present referred to as "Factor III".

The object of the present paper is to record the actual conditions in which growth has been obtained. In subsequent papers the various classes of nutrients will be discussed in detail.

AËROBIC GROWTH OF STAPHYLOCOCCUS.

The nutrients used for aërobic growth were as follows:

		Concentration in final medium.
KH_2PO_4	4·5 g.	..
Water	550 ml.	..
$N/1$ NaOH	26 „	..
*s-alanine	0·12 g.	$M/1500$†
s-valine	0·15 „	„
s-leucine	0·17 „	„
s-glycine	0·05 „	„
l-proline	0·07 „	„
l-oxyproline	0·08 „	„
s-aspartic acid	0·18 „	„
d-glutamic acid	0·09 „	„
s-methionine	0·07 „	$M/4000$
s-phenylalanine	0·08 „	„
l-tyrosine	0·05 „	„
d-arginine HCl	0·05 „	„
l-histidine HCl	0·05 „	„
s-lysine HCl	0·09 „	„

The amino-acids were dissolved, the pH brought to 7·4 and the volume adjusted to 600 ml. This basal mixture was distributed into tubes in 6 ml. volumes and autoclaved.

The following were autoclaved (or filtered) separately and added aseptically in the quantities indicated per tube:

Water	3·20 ml.
Ferrous ammonium sulphate $M/500$ in $N/50$ HCl (filtered)	0·25 „
Staphylococcus factor 0·2 mg./ml.	0·1 „
‡Dithiodiglycollic acid (Na salt) $\equiv M/10$ SH	0·2 „
Glucose $M/2$ (filtered)	0·25 „
	4·0 „

* s- denotes a substance of synthetic origin.

† Representing molarities of one only of the optical isomers of racemic mixtures.

‡ Prepared by oxidizing thiolacetic acid with H_2O_2 (Pirie, 1931), neutralizing, concentrating to a syrup and precipitating with alcohol.

The filter used was the glass filter using Seitz pads described by Knight and Fildes (1936). Some of the constituents could, of course, be added to the basal mixture and autoclaved. They were kept separate merely for experimental convenience.

The same rigid technique as formerly described (Knight and Fildes, 1933) was adopted with the object of excluding unknown constituents from the cultures. The inocula were reduced to the smallest dimensions. As a routine the smallest amount of an agar culture, not more than 24 hours old, which could be lifted with a platinum *point* was emulsified in 15 ml. water, and of this 1 drop was added per tube. The number of cocci was approximately 10,000, but 1/100 or less of this number were also capable of growing in the mixture though with a longer lag period. All experiments have been carried out with one culture isolated from a case of cellulitis and maintained on agar. The tubes were incubated in air at 37° C. in an upright position. The introduction of CO_2 into the atmosphere was unnecessary for the growth of this organism under the conditions used (Gladstone, Fildes and Richardson, 1935).

Most of the amino-acids in this mixture were included on general grounds, and as yet few of them have been shown to be indispensable. The list is approximately the same as that used by Fildes and Richardson (1935) for the growth of *Cl. sporogenes*. It will, however, be noted that serine, cystine and tryptophan were omitted; serine, because it was found at one stage of the work to be somewhat inhibitory, and cystine and tryptophan because they were unnecessary under the conditions of these experiments.

ANAËROBIC GROWTH OF STAPHYLOCOCCUS.

Anaërobic growth of the same culture was achieved with the medium described for aërobic growth with the following additions:

1. Pyruvic acid $M/1$ solution in water, filtered, 0·25 ml. added together with 0·5 ml. $M/2$ bicarbonate of soda solution also filtered. The stock pyruvic acid was prepared by the method of Clift and Cook (1932), and stored at 0° C. as a $5M$ solution in water.

2. Factor III. Most of the work has been carried out with preparations from yeast or other substances which gave the desired effect, namely anaërobic growth, but more recently confirmatory experiments have been made with the factor as a known chemical substance. A description of this is in the press (Richardson, 1936).

3. CO_2. For anaërobic growth the tubes were incubated in an anaërobic jar containing 5 p.c. of CO_2 in hydrogen, the pH being adjusted by including in the medium 0·5 ml. per tube of $M/2$ bicarbonate of soda solution.

It may be pointed out that when a facultative anaërobe such as *Staphylococcus aureus* is under investigation the distinction between aërobic and anaërobic growth is much more difficult than in the case of *Cl. sporogenes*, for instance, which can utilize only one of these mechanisms. When traces of oxygen are present in a mixture suitable for the anaërobic growth of *Cl. sporogenes*, so long as the concentration of oxygen is not too great, growth will proceed anaërobically, *i.e.* the organism will utilize a hydrogen acceptor

other than oxygen, namely glycine or proline. On the other hand, if a similar experiment is carried out with staphylococcus, the trace of oxygen will act as hydrogen acceptor and produce a trace of growth, apparently anaërobic but actually aërobic, although in fact the mixture used is not adequate for anaërobic growth. The anaërobic apparatus used was the McIntosh and Fildes anaërobic jar which was exhausted three times on the Geryk pump, filled with hydrogen containing 5 p.c. CO_2, and the platinized asbestos capsule heated for half an hour. Anomalous results were apt to be observed in the indispensability of "Factor III", especially if the jars were opened for examination and reincubated.

The growth under both aërobic and anaërobic conditions is first visible to the eye in about 17 hours, and reaches its maximum in 40–48 hours. The amount of growth is of the order of that obtained with peptone broth without glucose.

SUMMARY.

A nutrient mixture is described which is adequate for the aërobic or anaërobic growth of an untrained strain of *Staphylococcus aureus*.

REFERENCES.

CLIFT, F. P., AND COOK, R. P.—(1932) *Biochem J.*, **26**, 1788.
FILDES, P., AND RICHARDSON, G. M.—(1935) *Brit. J. exp. Path.*, **16**, 326.
GLADSTONE, G. P., FILDES, P., AND RICHARDSON, G. M.—(1935) *Ibid.*, **16**, 335.
KNIGHT, B. C. J. G.—(1935) *Ibid.*, **16**, 315.
Idem AND FILDES, P.—(1933) *Ibid.*, **14**, 112.
Idem.—(1936) *Biochem. J.*, **30**, 1138.
PIRIE, N.W.—(1931) *Ibid.*, **25**, 1565.
RICHARDSON, G. M.—(1937) *Ibid.* (in the press).

INDEX TO AUTHORS.

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